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14. ABSTRACT

We have previously shown anti-proliferative and pro-apoptotic effects of I3C and DIM through the NF-κB pathway in prostate cancer cells. To further explore the molecular mechanisms involved in the regulation of NF-κB by I3C/DIM, we investigated the effects of B-DIM, a formulated DIM with greater bioavailability, on AR, Akt, and NF-κB signaling in hormonesensitive LNCaP and hormone-insensitive C4-2B prostate cancer cells. We found that B-DIM significantly inhibited cell growth and induced apoptosis in both cell lines. By Akt transfection, RT-PCR, Western Blot analysis, and EMSA, we found that there could be a crosstalk between Akt, NF-κB, and AR in cell signaling. Importantly, we found that B-DIM significantly inhibited Akt activation, NF-κB DNA binding activity, and the expressions of AR and PSA, interrupting the crosstalk. Moreover, our confocal image study revealed that B-DIM inhibited AR nuclear translocation, leading to the down-regulation of AR target genes including PSA. These results suggest that B-DIM could inhibit cell growth and induce apoptosis partly through downregulation of AR, Akt and NF-κB signaling. These results along with our previous findings suggest that I3C and DIM may be potent agents for the prevention and/or treatment of androgen sensitive and androgen-refractory prostate cancers.

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Introduction

Previous epidemiological and dietary studies have shown an association between high dietary intakes of vegetables and decreased prostate cancer risk (1). Among vegetables with anticarcinogenic properties, the cruciferous vegetable family appears to be the most effective at reducing the risk of cancers (2). Indole-3-carbinol (I3C), a common phytochemical in the human diet, is present in almost all members of the cruciferous vegetable family, and it is readily converted to its dimeric product, 3,3'-diinolylmethane (DIM). It has been found that I3C and DIM inhibit the carcinogenesis in animal experiments and the growth of various cancer cells in culture (3, 4). The data from our laboratory and others have shown that I3C induces apoptotic cell death in cancer cells by up-regulation of Bax and $p21^{WAF1}$ and down-regulation of Bcl-2 and Bcl_{XL} (5, 6). We have found that I3C and DIM regulate the expression of genes which are critically involved in the control of cell growth, cell cycle, apoptosis, signal transduction, Phase I and II enzymes, and oncogenesis (7). We have also observed a drastic reduction in the activated form of the Akt (6, 8), and DNA-binding activity of NF-κB in prostate cancer cells treated with I3C or DIM (5, 8), suggesting the effects of I3C and DIM on Akt and NF-κB pathways which are two of the important pathways in cancer cells. Because AR interaction with Akt and NF-κB pathways plays important roles in the development and progression of prostate cancer, we hypothesize that there is a crosstalk between Akt, NF-κB, and AR signaling pathways and that I3C and DIM may inhibit the activity of Akt and NF-κB and, in turn, down-regulate AR and PSA, leading to the induction of apoptotic processes. To test our hypotheses we investigated how Akt, NF-κB, and AR pathways may cross-talk and how I3C and DIM inhibit Akt, NF-κB, and AR activation leading to apoptotic cell death in androgen sensitive and insensitive prostate cancer cells. We have also investigated the effects of I3C and DIM on other NF-κB upstream signaling molecules such as MEKK, MEK, NIK and IKK, which are known to play important roles in the activation of NF-κB. The results of this study will provide us with molecular mechanisms of action of I3C and DIM in prostate cancer cells. It will also provide us with molecular markers that may be useful for monitoring the efficacy of I3C and DIM during *in vivo* animal or human studies. More importantly, these results should identify novel pathways, which could be targeted by I3C and DIM for the development of combinational therapeutic approaches for the prevention and/or treatment of androgen sensitive and hormone-refractory prostate cancers.

Body of Report

The original statement of work in the proposal is listed below:

Task-1: We will determine whether treatment of prostate epithelial cells with I3C/DIM elicit responses leading to the modulation of NF-κB and investigate the molecular mechanism of NF-κB inactivation as proposed under specific aim-1. This investigation will be conducted using both I3C as well as DIM in androgen sensitive (LNCaP) and androgen independent (PC-3) prostate cancer cells and the data will be compared to those obtained from non-tumorigenic prostate epithelial cells (CRL2221). Task 1 will take 8 months to complete. Time Period 0-8 months.

Task-2: We will determine whether constitutive activation of Akt (by gene transfection studies) increases NF-κB activation in prostate epithelial cells as indicated under task-1 and thereby inhibits apoptotic processes induced by I3C/DIM. We will conduct transient transfection experiments followed by establishment of stably transfected cells in the future in order to determine the inhibition of I3C/DIM induced cell death in those cells that over-express Akt and NF-κB. Task 2 will take 6-12 months to complete. Time Period 8-20 months.

Task-3: Once we complete task-1 and 2, we will start working on task 3, which will have a significant number of transfection experiments. Task 3 will determine whether treatment of prostate epithelial cells with I3C/DIM will elicit responses that may determine causal association between the inactivation of Akt and NF-κB with the induction of apoptosis. Furthermore, we will investigate which signaling pathways are modified by I3C/DIM that lead to NF-κB inactivation. We will investigate different kinases that are involved in the NF-κB pathway, particularly the status of IKK, MAP kinase-MEK, MEKK1 and NIK. In order to establish cause and effect relationships of these genes with the ultimate effect of I3C/DIM, several transfection experiments are planned which will be very time consuming and labor intensive. Hence, this task will take considerably more time. We expect to complete this task within 12 months. Time Period 20-32 months.

Task-4: Task-4 will be focused on completing all data analysis, manuscript writing and for the development of novel ideas that may be submitted to Federal Agencies for further continued funding in a larger scale encompassing animal and human investigations to test (a) whether I3C/DIM could be an effective agent for the prevention and/or for the treatment of tumors in animal models, (b) whether I3C/DIM pre-exposure of prostate cancer cells will sensitize these cells to commonly available chemotherapeutic agents, and finally (c) whether I3C/DIM could be an effective agent for the prevention and/or treatment of human prostate cancer. Task 4 will be completed within the time period of 32-36 months.

In the last annual report, we provided evidence in support of the research accomplishments that we made last year and indicated that (a) the task-1 and task-2 have been fully completed; and (b) we have completed certain parts of task-3 and indicated that the remaining experiments for task-3 and task-4 will be completed next year.

We are now reporting the research accomplishments associated with task-3 and task-4 as outlined in the Statement of Work.

Task-3. Our task 3 was to determine whether treatment of prostate cancer cells with I3C and DIM will elicit responses that may determine causal association between the inactivation of Akt and NF-κB with the induction of apoptosis. The task 3 was also to investigate what signaling pathways are modified by I3C/DIM that lead to Akt and NF-κB inactivation and apoptosis. Because AR interacts with Akt and NF-κB pathways and plays important roles in the development and progression of prostate cancer, we have further investigated the effects of B-DIM, a specially formulated DIM with greater bioavailability, on AR, Akt, and NF-κB in hormone sensitive LNCaP cells (AR positive) and hormone insensitive C4-2B cells, which are also AR positive and represent a majority of hormone-refractory prostate cancers in men. These investigations are important for further elucidating the mechanistic role of NF-κB signaling pathway during I3C/DIM induced cell death. Therefore, we have conducted mechanistic experiments which were not included in task-3 but become necessary for continuation of our investigation in order to attract further funding from the NIH.

A. B-DIM significantly inhibited the growth of LNCaP and C4-2B cells.

Prior to our experiment, we tested the effect of androgen (DHT) on the growth of LNCaP and C4-2B cell lines. We found that LNCaP cells, which are known to be sensitive to androgen, showed growth stimulation of 25% and 35% at 48 and 72 hours, respectively. In contrast, C4-2B, which are insensitive to androgen, showed minimal or no growth stimulation. Importantly, we found that the treatment of LNCaP and C4-2B prostate cancer cells with B-DIM resulted in a dose- and time-dependent inhibition of cell proliferation (Figures 1 and 2) with maximal inhibition seen at 50 μM, demonstrating a potent growth inhibitory effect of B-DIM on both LNCaP and C4-2B cells.

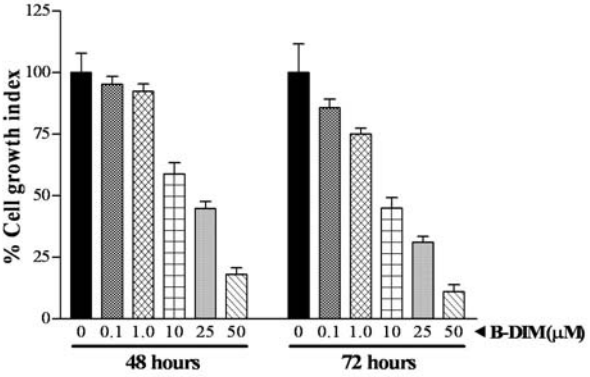

Figure 1: *MTT assay showing the significant inhibition of cell growth by B-DIM in LNCaP cells.*

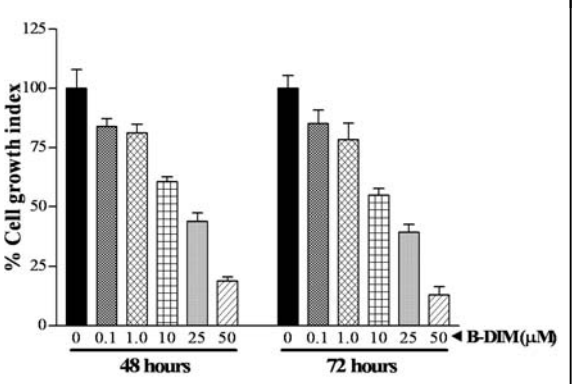

Figure 2: *MTT assay showing the significant inhibition of cell growth by B-DIM in C4-2B cells.*

B. B-DIM significantly induced apoptosis in LNCaP and C4-2B cells.

By apoptosis ELISA assay, we observed a significant induction of apoptosis in both LNCaP and C4-2B prostate cancer cells (Figures 3 and 4). This induction of apoptosis was time-dependent and directly correlated with the inhibition of cell growth. These data suggest that the growth inhibitory activity of B-DIM was partly attributed to an increase in apoptotic cell death.

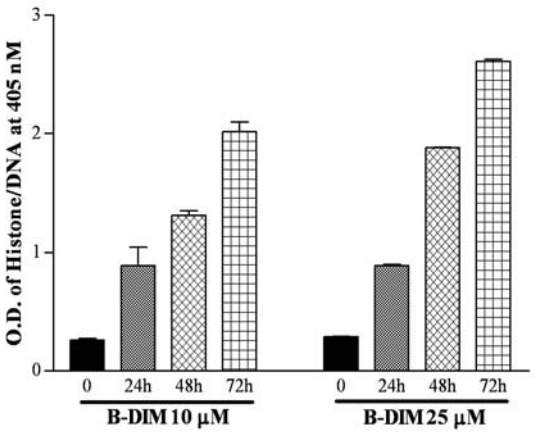

Figure 3: *ELISA showing the significant induction of cell apoptosis by B-DIM in LNCaP cells.*

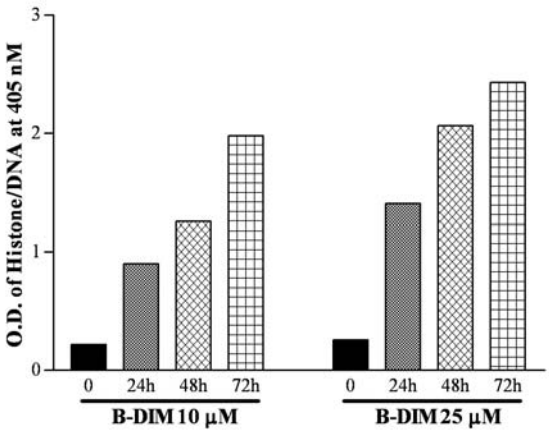

Figure 4: *ELISA showing the significant induction of cell apoptosis by B-DIM in C4-2B cells.*

C. B-DIM significantly inhibited AR and PSA mRNA expression in LNCaP and C4-2B cells.

Because AR and PSA play important roles in the development and progression of prostate cancer, we detected the effects of B-DIM on AR and PSA mRNA expressions by RT-PCR. We found that B-DIM significantly inhibited mRNA expression levels of AR and PSA in both LNCaP and C4-2B prostate cancer cells (Figures 5-8).

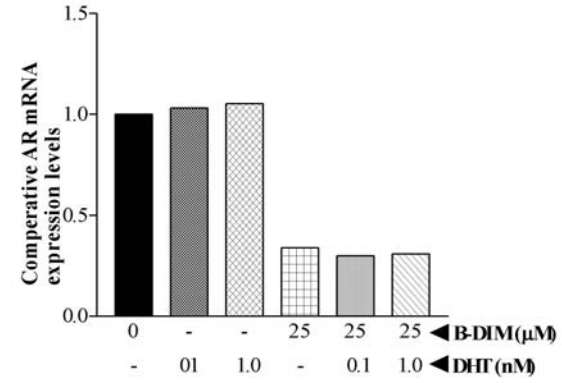

Figure 5: *RT-PCR analysis showing the significant inhibition of AR mRNA expression by B-DIM in LNCaP cells.*

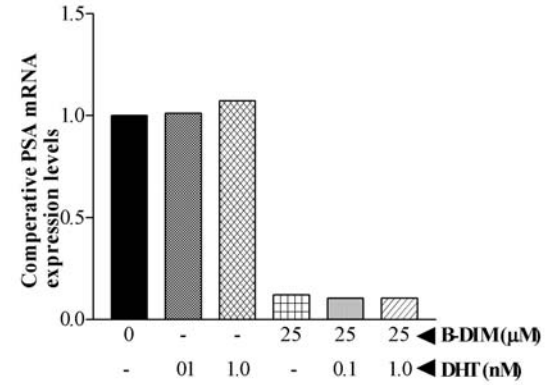

Figure 6: *RT-PCR analysis showing the significant inhibition of PSA mRNA expression by B-DIM in LNCaP cells.*

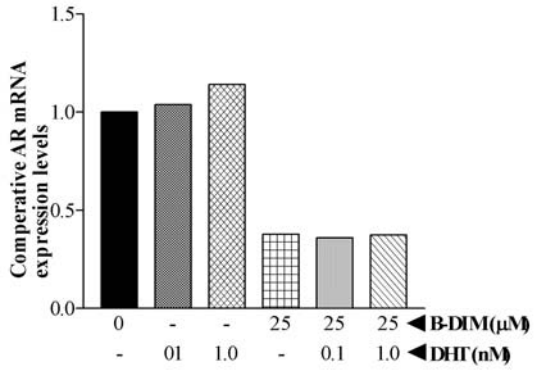

Figure 7: *RT-PCR analysis showing the significant inhibition of AR mRNA expression by B-DIM in C4-2B cells.*

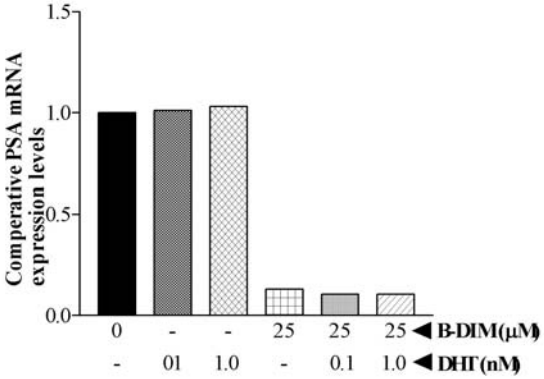

Figure 8: *RT-PCR analysis showing the significant inhibition of PSA mRNA expression by B-DIM in C4-2B cells.*

By PSA promoter-luciferase vector transfection assay, we also found that B-DIM significantly inhibited PSA promoter activity in LNCaP and C4-2B cells (Figure 9), and these results are consistent with the inhibition of PSA mRNA expression as observed by RT-PCR analysis.

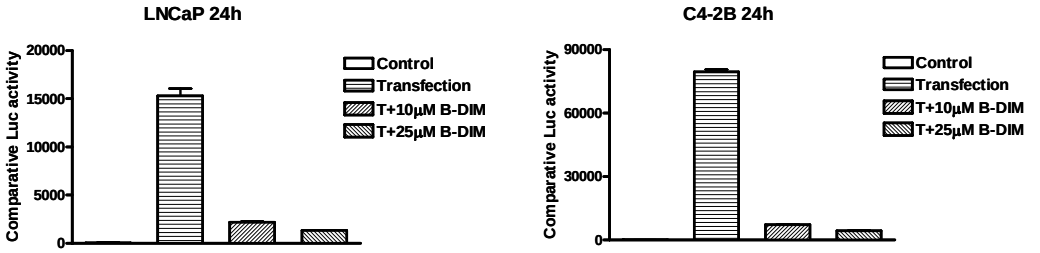

Figure 9: *PSA-Luc transfection and luciferase assay showing the significant inhibition of PSA promoter activity by B-DIM in LNCaP and C4-2B cells (T: Transfection).*

D. B-DIM significantly inhibited AR and PSA protein expression in LNCaP and C4-2B cells.

We also conducted Western Blot analysis to test if the down-regulation of AR and PSA mRNA expression by B-DIM leads to the decrease in the protein expression levels of AR and PSA. We found that B-DIM significantly inhibited the expression levels of AR and PSA proteins in both LNCaP and C4-2B prostate cancer cells in a time-dependent as well as dose-dependent manners (Figures 10 and 11).

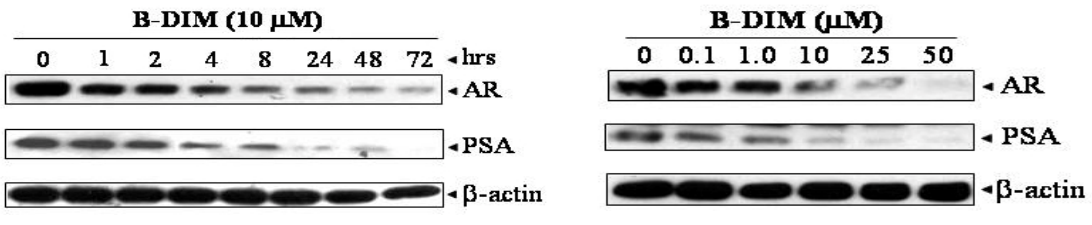

Figure 10: *Western Blot analysis showing the significant inhibition of AR and PSA protein expression by B-DIM in LNCaP cells in time- and dose-dependent manners.*

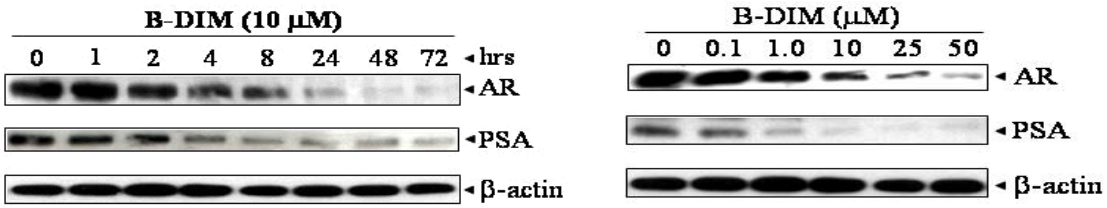

Figure 11: *Western Blot analysis showing the significant inhibition of AR and PSA protein expression by B-DIM in C4-2B cells in time- and dose-dependent manners.*

E. B-DIM significantly inhibited AR translocation to the nucleus in LNCaP and C4-2B cells.

It has been well known that AR translocates into the nucleus and binds to its target genes, regulating the expression of target genes, i.e. PSA. To investigate if B-DIM regulates the expression of PSA through inhibition of AR nuclear translocation, we investigated AR location before and after B-DIM treatment by immunostaining and confocal imaging. We found that B-DIM inhibited AR nuclear translocation in LNCaP and C4-2B cells (Figure 12).

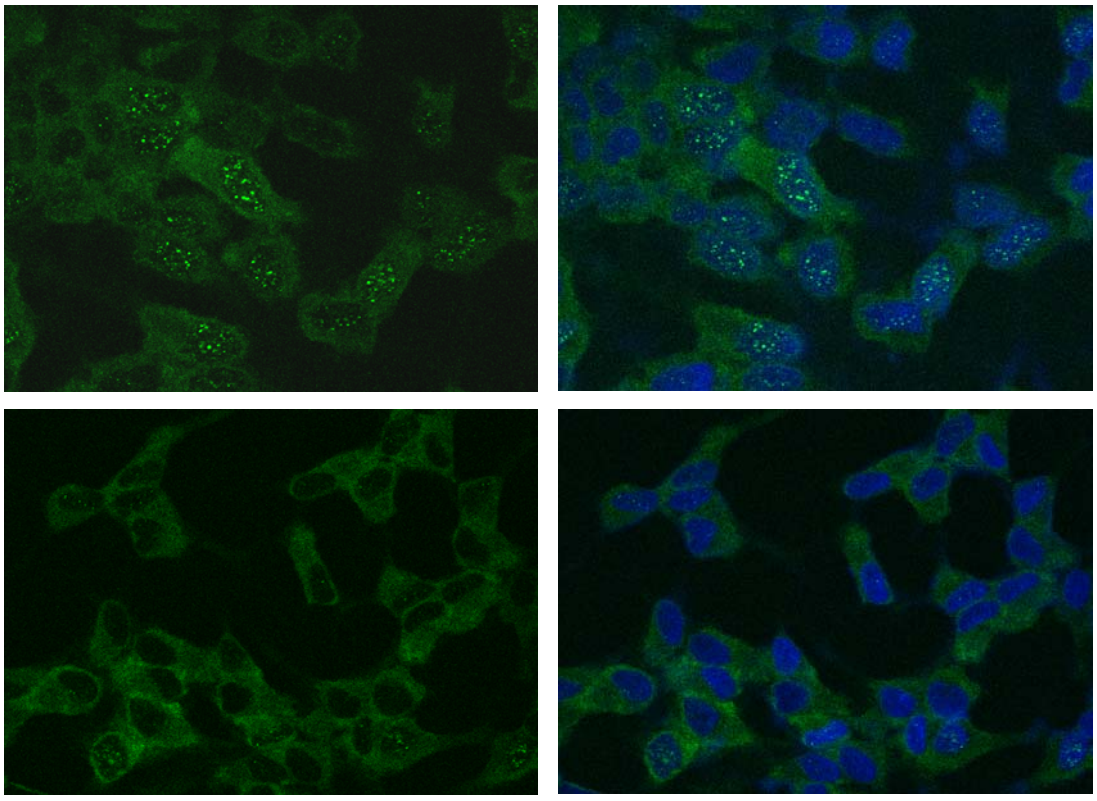

Figure 12: *Confocal imaging showing that B-DIM inhibited AR nuclear translocation in LNCaP cells (Upper panel: Control; Lower panel: B-DIM treatment).*

F. Down-regulation of Akt by B-DIM led to the inhibition of NF-κB, AR, and PSA.

By Western Blot analysis, we found that B-DIM down-regulated the protein levels of p-Akt, nuclear NF-κB, AR, and PSA in both LNCaP and C4-2B cells (Figure 13). To further investigate the relationship between Akt, NF-κB, AR, and PSA, and the effects of B-DIM on these molecules, we co-transfected Akt cDNA and NF-κB-Luc into LNCaP and C4-2B cells. We found that p-Akt, nuclear NF-κB, AR, and PSA were up-regulated after wild type Akt and Myr Akt transfections in LNCaP and C4-2B cells. However, the up-regulations of p-Akt, nuclear NF-κB, AR, and PSA by Akt transfection were significantly abrogated in both LNCaP and C4-2B cells treated with B-DIM (Figure 13).

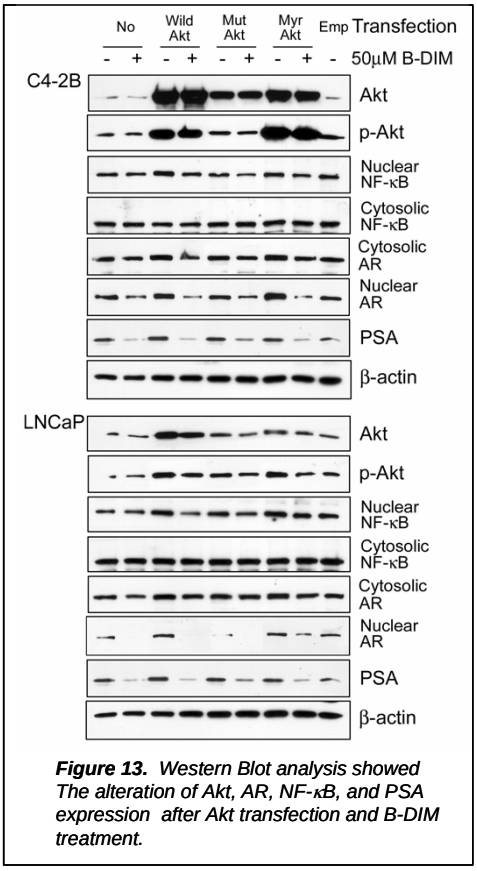

Figure 13. *Western Blot analysis showed The alteration of Akt, AR, NF-κB, and PSA expression after Akt transfection and B-DIM treatment.*

By transfection and luciferase assay, we found that luciferase activity was significantly increased after co-transfection with NF-κB-Luc and wild type Akt or Myr Akt in both LNCaP and C4-2B cells (Figure 14), suggesting the activation of NF-κB by Akt transfection. Moreover, B-DIM treatment significantly abrogated the up-regulation of luciferase activity caused by Akt transfection. Furthermore, we conducted EMSA to test NF-κB DNA binding activity in Akt transfected cells. The results showed that NF-κB DNA binding activity was significantly increased by wild type Akt and Myr Akt transfection and decreased by B-DIM treatment (Figure 15), which is consistent with the data from luciferase assay. Moreover, we also found that B-DIM at 10 or 25 μM significantly inhibited NF-κB DNA binding activity in the presence and absence of DHT in both LNCaP (Figure 16A and B) and C4-2B cells (Figure 16C and D).

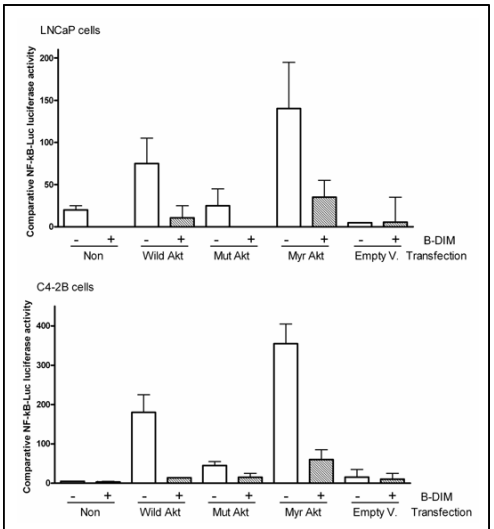

Figure 14. *Akt transfection and luciferase assay showed that B-DIM inhibited NF-κB DNA binding activity.*

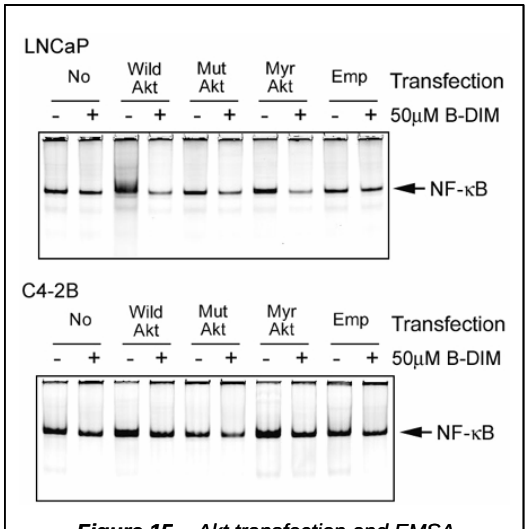

Figure 15. *Akt transfection and EMSA showed that B-DIM inhibited NF-κB DNA binding activity.*

Combined with the alteration of AR observed in Akt transfection study, our results suggest that there could be a crosstalk between p-Akt, NF-κB, and AR, and that inhibition of Akt activation by B-DIM could lead to the down-regulation of NF-κB, AR, and PSA. These results clearly suggest that B-DIM could inhibit cell growth and induce apoptosis partly though the down-regulation of AR, Akt, and NF-κB. Thus, we believe that B-DIM could be a potential therapeutic agent against both androgen-dependent and androgen-independent prostate cancers. These results will be submitted to "Journal of Biological Chemistry" for publication, and parts of the results have been presented in 96th AACR Annual Meeting (9).

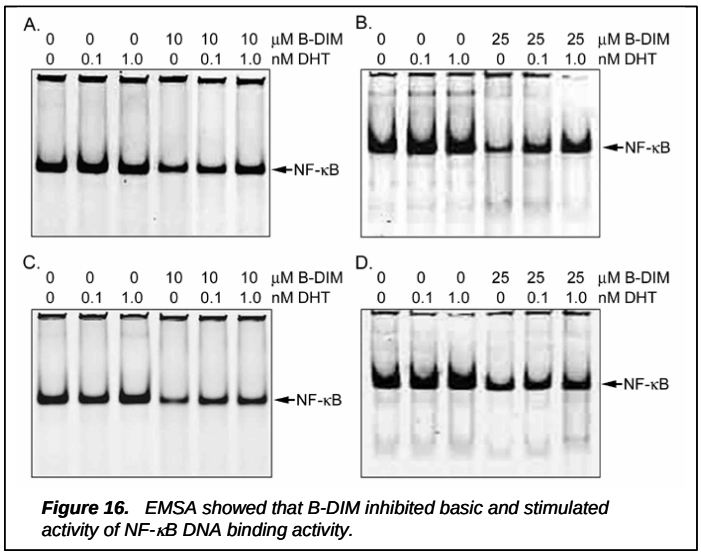

Figure 16. *EMSA showed that B-DIM inhibited basic and stimulated activity of NF-κB DNA binding activity.*

G. DIM regulated the molecules in the Akt pathway.

It has been reported that activated Akt regulates transcription through modulation of the activity of FOXO3a by phosphorylating it at three conserved serine/threonine resides. This leads to the prevention of FOXO3a into the nucleus, inhibiting expression of apoptosis-related genes. Activated Akt has also been found to phosphorylate and transactivate AR. Recent study showed that FOXO3a could also induce the expression of AR to protect cells from apoptosis when PI3K/Akt signal pathway was blocked by LY294002. These results suggest the importance of FOXO3a in Akt pathway. Therefore, we further investigated the effects of I3C/DIM on FOXO3a. The following are our findings:

1. DIM significantly inhibited Akt phosphorylation in hormone sensitive and insensitive prostate cancer cells regardless of AR status.

By Western Blot analysis, we found that DIM significantly inhibited the phosphorylation of Akt at Ser473 in hormone insensitive PC-3 (AR negative), hormone sensitive LNCaP (AR positive), and hormone insensitive C4-2B (AR positive) cells (Figure 17).

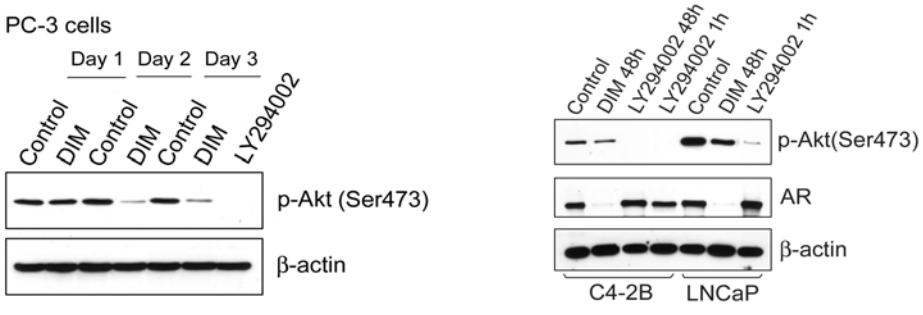

Figure 17. *Western Blot analysis showed that the level of phospho-Akt was significantly down-regulated by DIM treatment in PC-3, LNCaP, and C4-2B cells.*

2. DIM significantly inhibited FOXO3a phosphorylation.

By Western Blot analysis, we found that DIM did not alter the level of total FOXO3a. However, the level of pFOXO3a (Ser253) was significantly down-regulated by DIM treatment for 1 to 2 days in LNCaP and C4-2B cells (Figure 18).

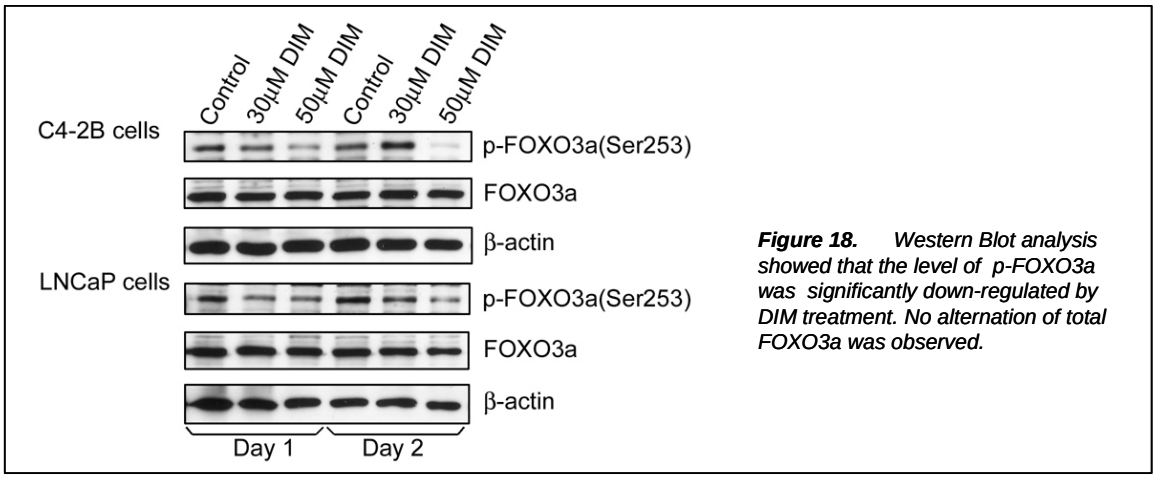

Figure 18. *Western Blot analysis showed that the level of p-FOXO3a was significantly down-regulated by DIM treatment. No alternation of total FOXO3a was observed.*

3. DIM inhibited FOXO3a binding to AR promoter DNA sequence.

By EMSA using AR promoter specific DNA oligos, we found that DIM significantly inhibited FOXO3a binding to the DNA oligos which include FOXO3a binding site in AR promoter (Figure 19).

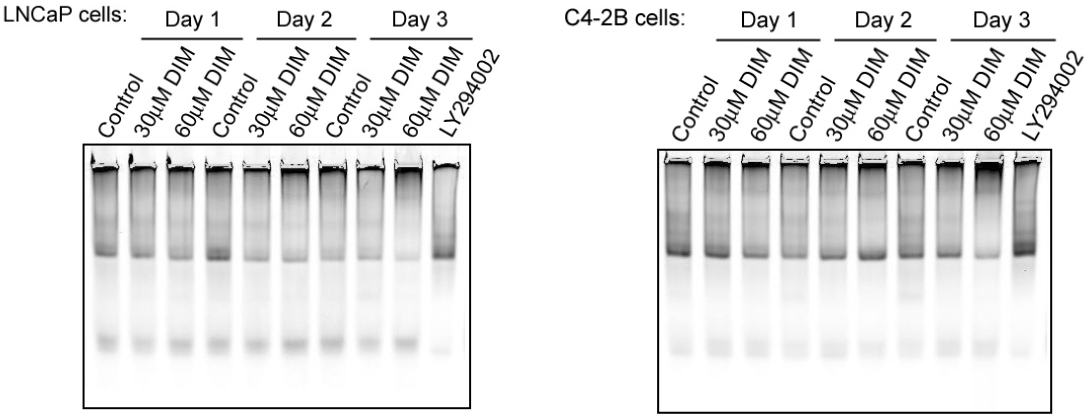

Figure 19. *EMSA showed that the DNA binding activity of FOXO3a was down-regulated by DIM treatment in LNCaP and C4-2B cells.*

To further confirm this result, we conducted ChIP assay to test binding activity of FOXO3a to AR promoter in LNCaP and C4-2B cells exposed to DIM and B-DIM. We found that DIM and B-DIM inhibited FOXO3a binding to AR promoter (Figure 20), consistent with our EMSA data.

These results suggest that DIM could inhibit Akt activation and subsequent phosphorylation of FOXO3a, resulting in the up-regulation of apoptosis-related gene expression and the induction of apoptosis in prostate cancer cells. DIM could also inhibit FOXO3a binding to AR promoter, leading to the down-regulation of AR expression. Therefore, DIM could be a potent inhibitor for both Akt and AR pathways.

Our published review articles (Cancer Research: Appendices 2; Book chapter: Appendices 4) are also attached to provide additional results that we have obtained from investigating effects of I3C/DIM on Akt and NF-κB pathways.

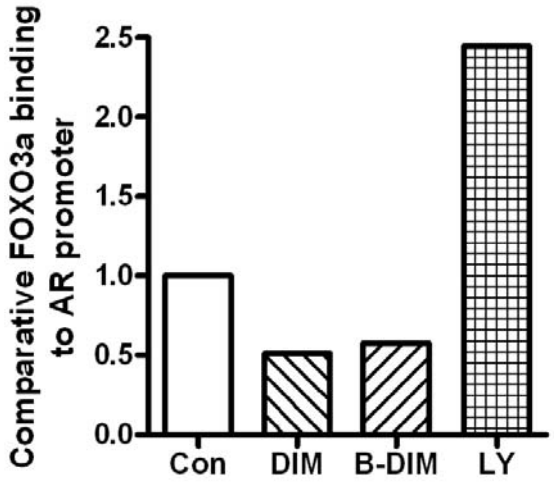

Figure 20. *ChIP assay showed that DIM and B-DIM inhibited FOXO3a binding to AR promoter in C4-2B cells.*

H. DIM enhanced anti-tumor activity of Taxotere.

The activation of Akt and NF-κB is believed to be responsible for resistance of cancer cells to chemotherapeutic agents, which is the major cause for treatment failure in cancer chemotherapy. Inhibition of NF-κB or Akt may potentiate the anticancer effect of chemotherapeutic agents. We have found that DIM inhibited the activation of Akt and NF-κB and induced apoptosis, suggesting that DIM may sensitize cancer cells to apoptotic cell death induced by chemotherapeutic agents. By cell growth inhibition assay, we found that combination treatment with DIM and Taxotere significantly inhibited the growth of LNCaP, C4-2B, and PC-3 prostate cancer cells compared to monotreatment (Figure 21). These results suggest that DIM could potentiate the anti-cancer effect of Taxotere partly due to inactivation of Akt and NF-κB.

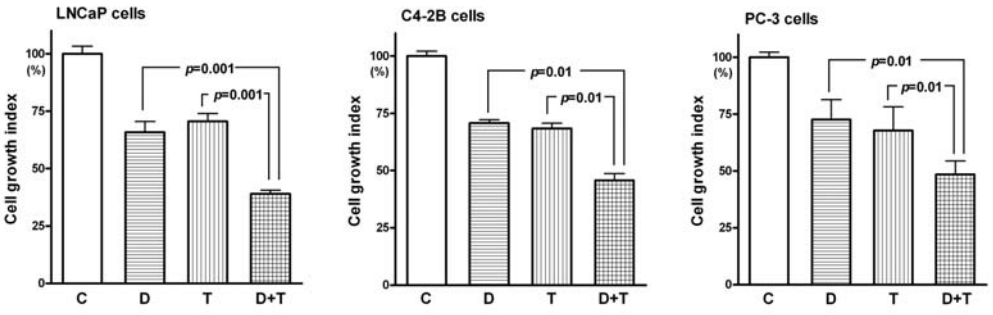

Figure 21. *Growth inhibition assay showed that combination treatment with DIM and Taxotere for 72h significantly inhibited the growth of LNCaP, C4-2B, and PC-3 cells compared to monotreatment.*

These results suggest that our strategies to sensitize prostate cancer cells to chemotherapeutic agent induced killing of prostate cancer cells by DIM is a novel breakthrough,

which could be used for devising novel therapeutic strategies for prostate cancer. Thus, DIM could be a potent agent for prevention and/or treatment of prostate cancer.

Conclusion: We have completed certain parts of task-3; however, we still need to investigate different kinases that are involved in the NF-κB pathway, particularly the status of IKK, MAP kinase-MEK, MEKK1 and NIK by transfection studies. Because of the difficulty and the time consuming nature of these experiments, we plan to complete this task in the fourth year for which we have already received approval for no cost extension.

Task-4. Task-4 will be focused on completing all data analysis, manuscript writing and for the development of novel ideas that may be submitted to Federal Agencies for further continued funding to test (a) whether I3C/DIM could be an effective agent for the prevention and/or for the treatment of tumors in animal models, (b) whether I3C/DIM pre-exposure of prostate cancer cells will sensitize these cells to commonly available chemotherapeutic agents, and finally (c) whether I3C/DIM could be an effective agent for the prevention and/or treatment of human prostate cancer.

We have analyzed the data demonstrated above and written a manuscript. The manuscript will be submitted to "Journal of Biological Chemistry" for publication. The abstract in the manuscript is presented below.

Abstract: Previous studies from our laboratory and others have shown anti-proliferative and pro-apoptotic effects of 3,3'-diindolylmethane (DIM) in prostate cancer cells. However, the molecular mechanism of action of DIM has not been fully elucidated. To further reveal such a mechanism, we investigated the effects of B-DIM, a formulated DIM with greater bioavailability, on AR, Akt, and NF-κB signaling in hormone-sensitive LNCaP and hormone-insensitive C4-2B prostate cancer cells. We found that B-DIM significantly inhibited cell growth and induced apoptosis in both cell lines. By Akt transfection, RT-PCR, Western Blot analysis, and EMSA, we found that there could be a crosstalk between Akt, NF-κB, and AR. Importantly, B-DIM significantly inhibited Akt activation, NF-κB DNA binding activity, and the expressions of AR and PSA, interrupting the crosstalk. Moreover, our confocal image study revealed that B-DIM inhibited AR nuclear translocation, leading to the down-regulation of AR target genes. These results suggest that B-DIM could inhibit cell growth and induce apoptosis partly mediated through down-regulation of AR, Akt and NF-κB signaling. These observations provide a scientific rationale for devising novel therapeutic approaches for the treatment of hormone sensitive or hormone-refractory prostate cancer by using B-DIM, a non-toxic agent, with other therapeutics.

We have also submitted a grant proposal to NIH based on the data that we have generated so far to continue our research.

Conclusion: We have completed certain parts of task-4. We will make progress on task-4 to complete all experiments, data analysis and manuscript writing during the fourth year of funding.

Key Research Accomplishments

- Determined the inhibitory effect of B-DIM, a specially formulated DIM with greater bioavailability, on cell growth in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the effect of B-DIM on the induction of apoptosis in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the inhibitory effect of B-DIM on the expression of AR and PSA in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the inhibitory effect of B-DIM on the AR nuclear translocation in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells.
- Determined the cross-talk between Akt, NF-κB, and AR in androgen sensitive LNCaP and androgen insensitive C4-2B prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the inhibitory effects of B-DIM on Akt, NF-κB, and AR pathways in Akt transfected LNCaP and C4-2B prostate cancer cells by transfection, luciferase assay, and EMSA.
- Determined the down-regulation of Akt, NF-κB, and AR by B-DIM leading to the cell growth inhibition and apoptotic cell death in LNCaP and C4-2B cells.
- Determined the inhibitory effects of B-DIM on the phosphorylation of FOXO3a and FOXO3a binding to AR promoter.
- Determined the effects of DIM on the sensitization of LNCaP and C4-2B prostate cancer cells to cell death induced by a chemotherapeutic agent, Taxotere.

Reportable Outcomes

1. Sarkar FH, Li Y. Using chemopreventative agents to enhance the efficacy of cancer therapy. Cancer Res. 66(7): 3347-3350, 2006.
2. Sarkar FH, Li Y. Plant derived antioxidants. In: Singh KK, editor. Oxidative Stress, Disease and Cancer. (In press), 2006.
3. Bhuiyan MM, Li Y, Banerjee S, Ahmed F, Wang Z, Ali S, Sarkar FH. Downregulation of androgen receptor by DIM contributes to cell growth inhibition and induction of apoptosis in both hormone sensitive LNCaP and insensitive C4-2B prostate cancer cells. (Will be submitted to JBC)

Conclusions

We have found that DIM significantly inhibited the cell growth and induced apoptosis through down-regulation of AR and PSA expression at mRNA and protein levels in hormone-sensitive LNCaP and hormone-insensitive C4-2B prostate cancer cells. By Akt transfection, RT-PCR, Western Blot analysis, and EMSA, we found that there could be a crosstalk between Akt, NF-κB, and AR. Importantly, DIM significantly inhibited Akt activation, NF-κB DNA binding activity, and the expressions of AR and PSA, interrupting this crosstalk. Moreover, our confocal image study revealed that DIM inhibited AR nuclear translocation, leading to the down-regulation of AR target genes including PSA. We also found that DIM inhibited Akt activation and subsequent phosphorylation of FOXO3a, resulting in the induction of apoptosis in prostate cancer cells. DIM also inhibited FOXO3a binding to AR promoter, leading to the down-regulation of AR expression. DIM also enhanced the anti-tumor activity of Taxotere, one of the common chemotherapeutic agents. These results along with our previous findings suggest that DIM could be a potent agent for the prevention and/or treatment of hormone sensitive and hormone-refractory prostate cancer. The task-1 and task-2 have been fully completed. We have completed the majority parts of task-3 and task-4. We plan to complete task-3 and task-4 in the fourth year under no cost extension as approved by the DOD.

References

1. Cohen JH, Kristal AR, Stanford JL. Fruit and vegetable intakes and prostate cancer risk. J Natl Cancer Inst 92: 61-68, 2000.
2. Verhoeven DT, Goldbohm RA, van Poppel G, Verhagen H, van den Brandt PA. Epidemiological studies on brassica vegetables and cancer risk. Cancer Epidemiol Biomarkers Prev 5: 733-748, 1996
3. Nachshon-Kedmi M, Yannai S, Haj A, Fares FA. Indole-3-carbinol and 3,3'-diindolylmethane induce apoptosis in human prostate cancer cells. Food Chem Toxicol 41: 745-752, 2003.
4. Nwankwo JO. Anti-metastatic activities of all-trans retinoic acid, indole-3-carbinol and (+)-catechin in Dunning rat invasive prostate adenocarcinoma cells. Anticancer Res 22: 4129-4135, 2002
5. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH. Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 20: 2927-2936, 2001
6. Chinni SR, Sarkar FH. Akt inactivation is a key event in indole-3-carbinol-induced apoptosis in PC-3 cells. Clin Cancer Res 8: 1228-1236, 2002.
7. Li Y, Li X, Sarkar FH. Gene expression profiles of I3C- and DIM-treated PC-3 human prostate cancer cells determined by cDNA microarray analysis. J Nutr 133: 1011-1019, 2003.
8. Li Y, Chinni SR, Sarkar FH. Selective growth regulatory and pro-apoptotic effects of DIM is mediated by Akt and NF-κB pathways in prostate cancer cells. Front Biosci 10: 236-243, 2005
9. Sarkar FH, Bhuiyan MM, Pilder M, Li Y. Down regulation of androgen receptor (AR) and its target gene (PSA) expressions by B-DIM leads to sensitization of prostate cancer cells to Taxotere-induced apoptosis. Proc Am Assoc Cancer Res 46:abstr 466, 2005.

Appendices

Publications, abstracts, and manuscripts during the second year of funding:

1. Sarkar FH, Li Y. Using chemopreventative agents to enhance the efficacy of cancer therapy. Cancer Res. 66(7): 3347-3350, 2006.
2. Sarkar FH, Li Y. Plant derived antioxidants. In: Singh KK, editor. Oxidative Stress, Disease and Cancer. (In press), 2006.
3. Bhuiyan MM, Li Y, Banerjee S, Ahmed F, Wang Z, Ali S, Sarkar FH. Downregulation of androgen receptor by DIM contributes to cell growth inhibition and induction of apoptosis in both hormone sensitive LNCaP and insensitive C4-2B prostate cancer cells. (Will be submitted to JBC)

Review

Using Chemopreventive Agents to Enhance the Efficacy of Cancer Therapy

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Abstract

Emerging evidence suggests that cancer preventative agents might be combined with chemotherapy or radiotherapy for the more effective treatment of cancer. Recent studies suggest that genistein and other dietary compounds that prevent cancer may enhance the efficacy of cancer therapeutics by modifying the activity of key cell proliferation and survival pathways, such as those controlled by Akt, nuclear factor-κB, and cyclooxygenase-2. In this article, we summarize the findings of recent investigations of chemopreventive agents in combination with cancer treatment regimens. (Cancer Res 2006; 66(7): 3347-50)

Background

Conventional cancer therapies, including surgery, chemotherapy, and radiotherapy, as single modalities have a limited but important role in the overall treatment of most solid tumors. Thus, the strategies of cancer treatment using combined therapies or combined agents with distinct molecular mechanisms are considered more promising for higher efficacy, resulting in better survival. In recent years, more dietary compounds [i.e., genistein, 3,3′-diindolylmethane, indole-3-carbinol (I3C), curcumin, (−)-epigallocatechin-3-gallate (EGCG), resveratrol, etc.] have been recognized as cancer chemopreventive agents because of their anticarcinogenic activity (1). Moreover, these compounds also exert the antitumor activities through regulation of different cell signaling pathways. Therefore, common cancer therapies combined with these dietary compounds may exert enhanced antitumor activity through synergic action or compensation of inverse properties. The combination treatment may also decrease the systemic toxicity caused by chemotherapies or radiotherapies because lower doses could be used. In this short article, we review current knowledge of the effects and the molecular mechanisms of the combination treatments published thus far, to give a brief view on the new and emerging field for optimal treatment of cancer patients with better survival.

Antitumor Activity of Common Cancer Therapies Are Potentiated by Chemopreventive Agents

Recently, there has been a growing interest in investigating the effects of genistein and other chemopreventive agents on the inhibition of cancer cell growth in combination with chemotherapeutics or other common therapies. The number of publications regarding potentiated antitumor effects of cancer therapies by chemopreventive agents has dramatically increased in 2005, suggesting that novel combination treatments with common cancer therapies and chemopreventive agents are beginning to receive much attention in cancer research.

Potentiation of chemotherapeutic effects. The *in vitro* and *in vivo* studies from our laboratory and others have shown that the antitumor effects of chemotherapeutic agents could be enhanced by combination treatment with chemopreventive agents. We have reported that genistein *in vitro* potentiated growth inhibition and apoptotic cell death caused by cisplatin, docetaxel, doxorubicin, and gemcitabine in prostate, breast, pancreas, and lung cancers (2–4). We found that pretreatment of cancer cells with 15 to 30 μmol/L genistein before the treatment with lower doses of chemotherapeutic agents caused a significantly greater degree of growth inhibition and apoptotic cell death, suggesting that increased antitumor activities of chemotherapeutic agents with lower toxicity to normal cells could be achieved by introducing genistein into the chemotherapeutic strategy. To investigate whether these phenomena that we observed *in vitro* could also exit *in vivo*, we conducted animal studies. We found that dietary genistein could potentiate the antitumor activities of gemcitabine and docetaxel in a tumor model, resulting in more tumor cell killing and apoptotic cell death (2, 3). By *in vitro* and *in vivo* studies, we also found that genistein could sensitize diffuse large cell lymphoma to cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP) chemotherapy (5). These results suggest that genistein enhances antitumor activities of chemotherapeutic agents both *in vitro* and *in vivo* in multiple tumors.

Other investigators have reported similar observations showing that the antitumor effects of chemotherapeutics could be enhanced by genistein. Hwang et al. recently reported that the combination of genistein and 5-fluorouracil (5-FU) synergistically induced apoptosis in chemoresistant HT-29 colon cancer cells (6). Genistein was also shown to enhance necrotic-like cell death in HER-2 overexpressing breast cancer cells treated with Adriamycin (7). Tanos et al. found that 1 to 10 μg/mL genistein inhibited the growth of dysplastic and malignant epithelial breast cancer cells *in vitro*, and that the addition of tamoxifen has a synergistic/additive inhibitory effect on breast cancer growth (8). These effects were not modulated by estrogen receptor. In addition, genistein and its isoflavone analogues have been found to decrease the side effects of tamoxifen through P450-mediated pathways (9). Thesis results support our findings and suggest the beneficial effects of genistein on cancer chemotherapy.

In addition to genistein, other dietary chemopreventive agents, including curcumin, EGCG, resveratrol, I3C, proanthocyanidin, and vitamin D, have been shown to enhance the antitumor activities of chemotherapeutic agents. A recent report by Lev-Ari et al. showed that curcumin and celecoxib synergistically inhibited the growth of colorectal cancer cells (10). Curcumin also enhanced the antitumor activities of cisplatin, doxorubicin, and Taxol in HA22T/VGH hepatic cancer cells, HeLa cells, or CAOV3 and SKOV3 ovarian cancer cells (11–13). In addition, the combined curcumin and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)

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treatment increased the number of hypodiploid cells and induced DNA fragmentation in LNCaP cells, suggesting a potential use of curcumin to sensitize prostate cancer cells for TRAIL-mediated immunotherapy (14). It has been reported that EGCG and tamoxifen synergistically induced apoptosis and growth inhibition in MDA-MB-231 human breast cancer cells (15). EGCG could also chemosensitize resistant tumor cells to doxorubicin through an increase in the accumulation of doxorubicin in the tumors of human carcinoma xenograft model (16). It has been found that resveratrol from grapes could sensitize non-Hodgkin's lymphoma and multiple myeloma cells to paclitaxel-mediated apoptosis (17). Proanthocyanidin, another compound from grapes, has been reported to enhance doxorubicin-induced antitumor effect and reverse drug resistance in doxorubicin-resistant K562/DOX cells, breast cancer cells, and mouse tumor xenograft models (18, 19). We and others have found that combinations of I3C and cisplatin or tamoxifen cooperate to inhibit the growth of PC-3 prostate and MCF-7 breast cancer cells more effectively than either agent alone (20, 21). In addition, analogues of vitamin D were also shown to potentiate the antiproliferative effect of doxorubicin, cisplatin, and genistein *in vitro* (22). These results clearly show that dietary cancer preventive agents can potentiate antitumor activities of common chemotherapeutics.

Potentiation of radiotherapy. We have investigated the effect of the combination of genistein and radiation on PC-3 prostate cancer cells. We found that the combination of genistein and radiation showed enhanced inhibitory effects on DNA synthesis, cell growth, and colony formation *in vitro* (23). Furthermore, we found that genistein combined with radiation led to a greater control of the growth of the primary tumor and metastasis to lymph nodes than genistein or radiation alone, suggesting that genistein enhanced the radiosensitivity of PC-3 prostate cancer cells (24). A similar report by other investigators showed that genistein enhanced the radiosensitivity of cervical cancer cells through increased apoptosis, prolonged cell cycle arrest, and impaired damage repair (25). Genistein was also shown to enhance radiosensitivity in human esophageal cancer cells *in vitro* (26), suggesting that the enhancement of radiosensitivity by genistein is not cell type dependent. Apart from genistein, another important chemopreventive agent, curcumin, at a low concentration in combination with radiation showed significant enhancement to radiation-induced clonogenic inhibition and apoptosis in PC-3 prostate cancer cells (27). These reports show that the radiotherapy combined with chemopreventive agents can cause more growth inhibition and apoptotic cell death of various cancers compared with monotreatment.

Molecular Mechanisms of Combination Treatment

The molecular mechanisms by which chemopreventive agents potentiate the antitumor effects of cancer therapies have not been fully elucidated. It is known that chemotherapy and radiotherapy can induce drug resistance in cancer cells, resulting in treatment failure. The major culprits involved in the development of drug resistance are multidrug resistance gene, nuclear factor-κB (NF-κB), and Akt. We and other investigators have found that the enhanced antitumor effects by chemopreventive agents could be, in part, through the regulation of NF-κB, Akt, and cyclooxygenase-2 (COX-2) pathways, which play important roles in cell survival (Fig. 1). Chemopreventive agents could also sensitize cancer cells to apoptosis by regulating several important molecules (i.e., Bcl-2, Bcl-X_L, survivin, caspases, $p21^{WAF1}$, etc.) in the apoptotic pathway (Fig. 1).

Regulation of the Akt pathway. Akt pathway is an important cell signaling pathway involved in drug resistance. It has been found that genistein enhanced necrotic-like cell death with the significant inhibition of Akt activity in breast cancer cells treated with genistein and Adriamycin, suggesting that the enhanced growth inhibition of combination is through the inactivation of the Akt pathway (7). Reports from our laboratory and others also showed that activated Akt was inhibited by genistein combined with gemcitabine or radiation in pancreatic, cervical, and esophageal cancer cells, suggesting that enhancement of chemotherapeutic or radiation effects by genistein may be partially mediated by the Akt pathway (3, 25, 26). Bava et al. recently reported that curcumin down-regulated Taxol-induced phosphorylation of Akt, which interacts with NF-κB, suggesting that enhanced antitumor activity by curcumin is through the Akt and NF-κB pathways (12).

Regulation of NF-κB pathway. It has been known that many chemotherapeutic agents induce activity of NF-κB, which causes drug resistance in cancer cells (28). By *in vitro* and *in vivo* studies, we found that NF-κB activity was significantly increased by cisplatin, docetaxel, gemcitabine, and radiation treatment, and that the NF-κB inducing activity of these agents was completely abrogated by genistein pretreatment in prostate, breast, lung, and pancreatic cancer cells, suggesting that genistein pretreatment inactivates NF-κB and may contribute to increased growth inhibition and apoptosis induced by these agents (2–4, 23). We also found that genistein potentiated the antitumor activity of CHOP by inhibition of NF-κB in lymphoma cells (5). Similarly, curcumin has been found to inhibit the activity of NF-κB and sensitize cancer cells to cisplatin or Taxol-induced apoptosis (12, 29).

Regulation of apoptosis pathways. It has been reported that curcumin combined with cisplatin decreased the expression of several apoptosis-related genes, including *c-myc*, *Bcl-X_L*, *c-IAP-2*, *NAIP*, and *XIAP* (11). The combination of curcumin and TRAIL also induced cleavage of procaspase-3, procaspase-8, and procaspase-9; truncation of Bid; and release of cytochrome *c* from the mitochondria in prostate cancer cells, indicating that the apoptotic pathway is triggered in prostate cancer cells treated with combination of curcumin and TRAIL (14). We and others also found that genistein combined with docetaxel or gemcitabine significantly inhibited Bcl-2, Bcl-X_L, and survivin and induced $p21^{WAF1}$, suggesting that combination treatment regulates the important molecules in the apoptotic pathway (2, 3).

Regulation of other pathways. It has been found that the combination of 5-FU and genistein enhanced therapeutic effects in colon cancers through the COX-2 pathway (6). A recent report showed that curcumin or EGCG could down-regulate COX-2 expression without any change of COX-1 expression at both the mRNA and protein levels in colorectal or prostate cancer cells, suggesting that a combination of curcumin or EGCG with chemotherapeutic agents could be an improved strategy for the treatment of colorectal or prostate cancer (10, 30). Indeed, the synergistic growth inhibitory effect of curcumin and celecoxib was found in colorectal cancer cells through inhibition of the COX-2 pathway (10). Apart from the COX-2 pathway, the molecules in cell cycle regulation may also be involved in mechanisms of combination treatment. It has been reported that combination of I3C and tamoxifen caused a more pronounced decrease in cyclin-dependent kinase 2 (CDK2)–specific enzymatic activity, CDK6 expression, and

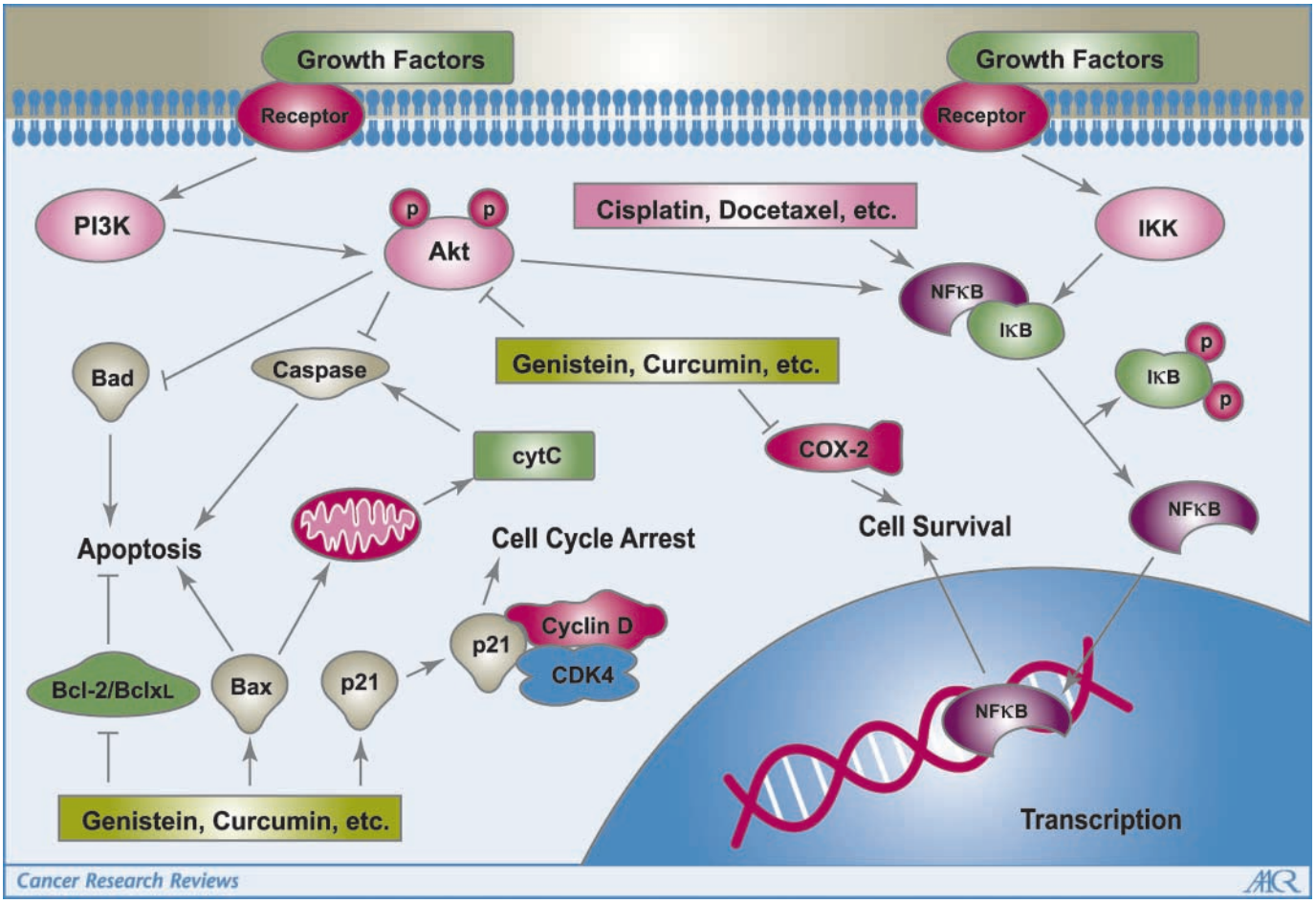

Figure 1. Chemopreventative agents enhance antitumor effects of chemotherapies and radiotherapies through the regulation of Akt, NF-κB, COX-2, and apoptosis pathways. *cytC,* cytochrome *c*.

the level of phosphorylated retinoblastoma protein (20). The enhanced effects of chemotherapy by chemopreventive agents may also be related to immunopotentiating activities through reduction of interleukin-6 (IL-6; ref. 13) and enhancements of lymphocyte proliferation, natural killer cell cytotoxicity, $CD4^+/CD8^+$ ratio, IL-2, and IFN-γ productions (18). In addition, genistein and its isoflavone analogues showed the potential to decrease side effects of tamoxifen through metabolic interactions that inhibit the formation of α-hydroxytamoxifen via inhibition of CYP1A2 (9), suggesting the beneficial effects of genistein in combination with tamoxifen.

Conclusion and Perspective

The *in vitro* and *in vivo* studies reviewed above all suggest that dietary chemopreventive agents may serve as potent agents for enhancing the therapeutic effects of chemotherapy, radiotherapy, or other standard therapeutics for the treatment of human cancers. However, further in-depth mechanistic studies, *in vivo* animal experiments, and clinical trials are needed to bring this concept into practice to fully appreciate the value of chemopreventive agents in combination therapy of human cancers.

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References

1. Surh YJ. Cancer chemoprevention with dietary phytochemicals. Nat Rev Cancer 2003;3:768–80.
2. Li Y, Ahmed F, Ali S, Philip PA, Kucuk O, Sarkar FH. Inactivation of nuclear factor kappaB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. Cancer Res 2005;65:6934–42.
3. Banerjee S, Zhang Y, Ali S, et al. Molecular evidence for increased antitumor activity of gemcitabine by genistein *in vitro* and *in vivo* using an orthotopic model of pancreatic cancer. Cancer Res 2005;65:9064–72.
4. Li Y, Ellis KL, Ali S, et al. Apoptosis-inducing effect of chemotherapeutic agents is potentiated by soy isoflavone genistein, a natural inhibitor of NF-kappaB in BxPC-3 pancreatic cancer cell line. Pancreas 2004;28:e90–5.
5. Mohammad RM, Al-Katib A, Aboukameel A, Doerge DR, Sarkar F, Kucuk O. Genistein sensitizes diffuse

large cell lymphoma to CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy. Mol Cancer Ther 2003;2:1361–8.
6. Hwang JT, Ha J, Park OJ. Combination of 5-fluorouracil and genistein induces apoptosis synergistically in chemo-resistant cancer cells through the modulation of AMPK and COX-2 signaling pathways. Biochem Biophys Res Commun 2005;332:433–40.
7. Satoh H, Nishikawa K, Suzuki K, et al. Genistein, a soy isoflavone, enhances necrotic-like cell death in a breast cancer cell treated with a chemotherapeutic agent. Res Commun Mol Pathol Pharmacol 2003;113-4:149–58.
8. Tanos V, Brzezinski A, Drize O, Strauss N, Peretz T. Synergistic inhibitory effects of genistein and tamoxifen on human dysplastic and malignant epithelial breast cells *in vitro*. Eur J Obstet Gynecol Reprod Biol 2002;102: 188–94.
9. Chen J, Halls SC, Alfaro JF, Zhou Z, Hu M. Potential beneficial metabolic interactions between tamoxifen and isoflavones via cytochrome P450-mediated pathways in female rat liver microsomes. Pharm Res 2004;21: 2095–104.
10. Lev-Ari S, Strier L, Kazanov D, et al. Celecoxib and curcumin synergistically inhibit the growth of colorectal cancer cells. Clin Cancer Res 2005;11:6738–44.
11. Notarbartolo M, Poma P, Perri D, Dusonchet L, Cervello M, D'Alessandro N. Antitumor effects of curcumin, alone or in combination with cisplatin or doxorubicin, on human hepatic cancer cells. Analysis of their possible relationship to changes in NF-kB activation levels and in IAP gene expression. Cancer Lett 2005; 224:53–65.
12. Bava SV, Puliappadamba VT, Deepti A, Nair A, Karunagaran D, Anto RJ. Sensitization of taxol-induced apoptosis by curcumin involves down-regulation of nuclear factor-kappaB and the serine/threonine kinase Akt and is independent of tubulin polymerization. J Biol Chem 2005;280:6301–8.
13. Chan MM, Fong D, Soprano KJ, Holmes WF, Heverling H. Inhibition of growth and sensitization to cisplatin-mediated killing of ovarian cancer cells by polyphenolic chemopreventive agents. J Cell Physiol 2003;194:63–70.
14. Deeb D, Xu YX, Jiang H, et al. Curcumin (diferuloyl-methane) enhances tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in LNCaP prostate cancer cells. Mol Cancer Ther 2003; 2:95–103.
15. Chisholm K, Bray BJ, Rosengren RJ. Tamoxifen and epigallocatechin gallate are synergistically cytotoxic to MDA-MB-231 human breast cancer cells. Anticancer Drugs 2004;15:889–97.
16. Zhang Q, Wei D, Liu J. *In vivo* reversal of doxorubicin resistance by (−)-epigallocatechin gallate in a solid human carcinoma xenograft. Cancer Lett 2004;208:179–86.
17. Jazirehi AR, Bonavida B. Resveratrol modifies the expression of apoptotic regulatory proteins and sensitizes non-Hodgkin's lymphoma and multiple myeloma cell lines to paclitaxel-induced apoptosis. Mol Cancer Ther 2004;3:71–84.
18. Zhang XY, Li WG, Wu YJ, et al. Proanthocyanidin from grape seeds potentiates anti-tumor activity of doxorubicin via immunomodulatory mechanism. Int Immunopharmacol 2005;5:1247–57.
19. Sharma G, Tyagi AK, Singh RP, Chan DC, Agarwal R. Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. Breast Cancer Res Treat 2004;85:1–12.
20. Cover CM, Hsieh SJ, Cram EJ, et al. Indole-3-carbinol and tamoxifen cooperate to arrest the cell cycle of MCF-7 human breast cancer cells. Cancer Res 1999;59:1244–51.
21. Sarkar FH, Li Y. Indole-3-carbinol and prostate cancer. J Nutr 2004;134:3493–8S.
22. Siwinska A, Opolski A, Chrobak A, et al. Potentiation of the antiproliferative effect *in vitro* of doxorubicin, cisplatin and genistein by new analogues of vitamin D. Anticancer Res 2001;21:1925–9.
23. Hillman GG, Forman JD, Kucuk O, et al. Genistein potentiates the radiation effect on prostate carcinoma cells. Clin Cancer Res 2001;7:382–90.
24. Hillman GG, Wang Y, Kucuk O, et al. Genistein potentiates inhibition of tumor growth by radiation in a prostate cancer orthotopic model. Mol Cancer Ther 2004;3:1271–9.
25. Yashar CM, Spanos WJ, Taylor DD, Gercel-Taylor C. Potentiation of the radiation effect with genistein in cervical cancer cells. Gynecol Oncol 2005;99:199–205.
26. Akimoto T, Nonaka T, Ishikawa H, et al. Genistein, a tyrosine kinase inhibitor, enhanced radiosensitivity in human esophageal cancer cell lines *in vitro*: possible involvement of inhibition of survival signal transduction pathways. Int J Radiat Oncol Biol Phys 2001;50:195–201.
27. Chendil D, Ranga RS, Meigooni D, Sathishkumar S, Ahmed MM. Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3. Oncogene 2004;23: 1599–607.
28. Chuang SE, Yeh PY, Lu YS, et al. Basal levels and patterns of anticancer drug-induced activation of nuclear factor-kappaB (NF-kappaB), and its attenuation by tamoxifen, dexamethasone, and curcumin in carcinoma cells. Biochem Pharmacol 2002;63:1709–16.
29. Venkatraman M, Anto RJ, Nair A, Varghese M, Karunagaran D. Biological and chemical inhibitors of NF-kappaB sensitize SiHa cells to cisplatin-induced apoptosis. Mol Carcinog 2005;44:51–9.
30. Hussain T, Gupta S, Adhami VM, Mukhtar H. Green tea constituent epigallocatechin-3-gallate selectively inhibits COX-2 without affecting COX-1 expression in human prostate carcinoma cells. Int J Cancer 2005;113: 660–9.

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36 Plant-Derived Antioxidants

Fazlul H. Sarkar and Yiwei Li

1. Introduction

As humans live in an aerobic environment, their exposure to reactive oxygen species (ROS) is continuous and unavoidable. The biological systems in the human body interact with the external environment to maintain an internal environment that favors survival, growth, differentiation, and reproduction. Although a number of defense systems have evolved to combat the accumulation of ROS, these defense systems are not always adequate to counteract the production of ROS, resulting in a state of oxidative stress.[1] It is important to note that oxidative stress has been linked to aging and a variety of chronic diseases such as atherosclerosis, neurodegenerative diseases, diabetes, pulmonary fibrosis, arthritis.[2,3] More importantly, oxidative stress could be carcinogenic because ROS can cause severe DNA damage, which plays an important role in carcinogenesis.[2,4] Once DNA damage occurs, DNA repair is a critical process in order to prevent mutagenesis. However, under oxidative stress, the repair of DNA damage can be inhibited by several redox-dependent metals, resulting in carcinogenesis.[4] Moreover, the activation of nuclear factor-kappa B (NF-κB) by ROS under oxidative stress has been known as a key event in carcinogenesis.[4] Therefore, antioxidants are important in combating cancers and some chronic diseases, which have been tightly linked with oxidative stress.

In nature, to resist oxygenic threat, antioxidants have evolved in parallel with our oxygenic atmosphere. Plants employ antioxidants to defend their structures against ROS produced during photosynthesis.[5] Plants, therefore,

produce various antioxidant components, which could be beneficial for human health. A variety of plant-derived components have been found to reduce oxidative stress via the antioxidant mechanism. Among them, isoflavones, curcumin, epigallocatechin-3-gallate, indole-3-carbinol (I3C), resveratrol, lycopene, vitamin E, and vitamin C have shown more promising effects on the reduction of oxidative stress.[6–12] Most of them have been found to inhibit NF-κB activation stimulated by ROS.[13–20] Moreover, these antioxidants have shown their inhibitory effects on atherosclerosis, neurodegeneration, oncogenesis, cancer growth, and metastasis,[21–24] suggesting that they could be used as chemopreventive and/or chemotherapeutic agents for some chronic diseases and cancers.

2. Oxidative Stress and NF-κB Activation in Chronic Diseases and Cancers

It has been well known that NF-κB activation stimulated by ROS is a very important event in the development of some chronic diseases and cancers,[25,26] which are linked with oxidative stress. Under the situation of oxidative stress, ROS induces DNA damage and alters cell signal transduction pathways including the NF-κB pathway.[27] The direct addition of H_2O_2 to culture medium activates NF-κB in many types of cell lines.[28] In addition, it has been found that ROS in cells is increased in response to the agents that also activate NF-κB.[28,29] These findings suggest that oxidative stress activates NF-κB activity in cells.

NF-κB plays important roles in the physiological processes as well as in the defensive response to injury, infection, and other stress conditions.[30] The NF-κB family is composed of several proteins: RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52), each of which may form homo- or heterodimers.[31,32] In human cells without specific extracellular signal, NF-κB is sequestered in the cytoplasm through tight association with its inhibitors: IκB, which acts as NF-κB inhibitor, and p100 proteins, which serve as both inhibitors and precursors of NF-κB DNA-binding subunits.[31,33] NF-κB can be activated by many types of stimuli including tumor necrosis factor-α (TNF-α), ultraviolet radiation, H_2O_2, free radicals, etc. The activation of NF-κB occurs through phosphorylation of IκB by

IKKβ and/or phosphorylation of p100 by IKKα, leading to degradation of IκB and/or the processing of p100 into a small form (p52). This process allows two forms of activated NF-κB (p50–p65 and p52–RelB) to become free, translocate into nucleus, bind to NF-κB-specific DNA-binding sites, and regulate downstream gene transcription.[33,34]

In this way, NF-κB controls the expression of many genes that are involved in cellular physiological processes including stress response, inflammation, differentiation, cell growth, apoptosis, etc.[35–37] The disorder of these physiological processes has been demonstrated to be linked with the occurrence of some chronic diseases and cancers. It has been reported that overexpression of NF-κB protects cells from apoptosis and favors cell survival, while inhibition or absence of NF-κB induces apoptosis.[38] An *in vivo* study showed that mice lacking NF-κB p65 died embryonically from extensive apoptosis in the liver, suggesting the anti-apoptotic role of NF-κB.[39] The deregulated cell proliferation or inability of cells to undergo apoptotic cell death results in the development of cancers. NF-κB also promotes the expression of genes related to inflammation and degeneration, resulting in chronic inflammatory and degenerative diseases.[25,40,41] Therefore, the deregulated NF-κB under oxidative stress has been described as a major cause in cancers and some of the chronic diseases.[26,40]

3. NF-κB as a Preventive or Therapeutic Target in Inflammatory Diseases and Cancers

Inhibition of NF-κB activation stimulated by ROS is now widely recognized as a valid strategy to combat inflammatory disease.[25,42] However, it has become obvious that inhibition of NF-κB activity is not only desirable for the treatment of inflammation but also in cancer therapy.[43,44] Examination of the inflammatory microenvironment in neoplastic tissues has supported the hypothesis that inflammation is a cofactor in oncogenesis for a variety of cancers. Many anti-inflammation drugs and antioxidants inhibit NF-κB activity and induce apoptosis; therefore, they may also be desirable in the treatment of cancers.

Experimental studies have shown the cellular growth and anti-apoptotic activity of NF-κB in malignant cells.[45,46] It has been reported that NF-κB

is constitutively activated in Hodgkin's tumor cells, whereas inhibition of NF-κB blocks the cell growth.[46] It has been demonstrated that NF-κB regulates growth and survival of multiple myeloma and that NF-κB is a novel therapeutic target in multiple myeloma.[47] Our data also showed that plant-derived antioxidant compounds including genistein, I3C, and 3,3′-diindolylmethane (DIM) inhibited the activity of NF-κB and the growth of cancer cells, and induced apoptosis in cancer cells,[13,17,48] suggesting that NF-κB is a target for cancer prevention and/or treatment.

Now it has become more obvious that inhibition of NF-κB activity is desirable in the prevention and treatment of cancers and inflammations. Thus, plant-derived antioxidants with NF-κB inactivation activity may serve as agents against cancers and some chronic diseases.

4. Plant-Derived Antioxidants Inhibiting Oxidative Stress and NF-κB Activation

4.1. *Isoflavones*

Isoflavones are a subclass of the more ubiquitous flavonoids and are much more narrowly distributed in soybeans. Genistein, daidzein, and glycitein are three main isoflavones found in soybeans. Genistein and daidzein have been found in relatively high concentration in soybeans and most soy-protein products, while much lower amounts of glycitein are present in soybeans. Experimental studies have revealed that isoflavones, particularly genistein, exert antioxidant effects on human cells. It has been reported that genistein protects cells against ROS by scavenging free radicals and reducing the expression of stress-response-related genes.[6,49] Isoflavones also stimulate antioxidant protein gene expression in Caco-2 cells.[50] Sierens *et al.*[51] have found that isoflavone supplementation reduces hydrogen peroxide-induced DNA damage in sperm, suggesting the antioxidant effects of isoflavone. In addition, it has been found that isoflavones and synthetic isoflavone derivatives suppress lipid peroxidation of human high-density lipoproteins, decrease oxidized low-density lipoproteins, and reduced atherosclerotic plaque thickness, suggesting their preventive and therapeutic effects on cardiovascular diseases.[52,53] Kawakami *et al.*[54] have also

reported that soy isoflavones may reduce the risk of some cardiovascular diseases through their radical scavenging function and hypocholesterolemic action. Moreover, it has been demonstrated that genistein inhibits tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate induced hydrogen peroxide production in human polymorphonuclear leukocytes and HL-60 cells, suggesting the inhibitory effect of genistein on carcinogenesis through antioxidant mechanism.[55]

4.1.1. *Inhibition of oxidative stress and NF-κB activation in vitro by soy isoflavone genistein*

Our laboratory has investigated whether genistein treatment could modulate NF-κB DNA binding activity in PC3 and LNCaP prostate cancer cells by electrophoresis mobility shift assay (EMSA). We found that 50 μM genistein treatment for 24–72 h significantly inhibited NF-κB DNA-binding activity in both cell lines.[13] We further investigated whether genistein could block NF-κB induction by oxidative stress inducers, H_2O_2 and TNF-α,[13] both of which have been previously shown to induce NF-κB DNA-binding activity. After treatment with H_2O_2 or TNF-α, we observed an increase in NF-κB DNA-binding activity in prostate cancer cell lines, as expected. However, when the cells were pre-treated with genistein for 24 h prior to stimulation with the inducing agent, genistein abrogated the induction of NF-κB DNA-binding activity elicited by either H_2O_2 or TNF-α. Western blot analysis of nuclear extracts showed similar results.[13] These results demonstrated that genistein not only reduced NF-κB DNA-binding activity in non-stimulated conditions, but inhibited NF-κB activation in cells under oxidative stress condition.

Other investigators also demonstrated similar effect of genistein on NF-κB in different types of cells. Baxa and Yoshimura[54] showed that genistein reduced NF-κB in T lymphoma cells via a caspase-mediated cleavage of IκBα. Tabary *et al.*[57] also found that genistein inhibited constitutive and inducible NF-κB activation and decreased interleukin-8 production in human cystic fibrosis bronchial gland cells. Our *in vitro* data along with results from other investigators suggested that genistein functions as an antioxidant, which could be a potent agent for the inhibition of oxidative stress and the prevention and/or treatment of cancers.

4.1.2. *Inhibition of oxidative stress and NF-κB activation in vivo by soy isoflavones*

Since our *in vitro* results showed inactivation of NF-κB by genistein treatment, we further investigated the effect of isoflavone supplementation on NF-κB activation *in vivo* in human volunteers.[14] The lymphocytes from healthy male subjects were harvested from peripheral blood and cultured for 24 h in the absence and presence of genistein. EMSA revealed that genistein treatment inhibited basal levels of NF-κB DNA-binding activity by 56% and abrogated TNF-α-induced NF-κB activity by 50%.[14] Furthermore, when human volunteers received 50 mg of soy isoflavone supplements (Novasoy™) twice daily for three weeks, TNF-α failed to activate NF-κB activity in lymphocytes harvested from these volunteers, while lymphocytes from these volunteers collected prior to soy isoflavone intervention showed activation of NF-κB DNA-binding activity upon TNF-α treatment *in vitro*.[14]

We further measured the levels of oxidative DNA damage in the blood of the six subjects before and after supplementation with Novasoy™. DNA was isolated from lymphocyte nuclei from the six subjects and analyzed for levels of 5-OHmdU, a modified DNA base that represents the endogenous status of cellular oxidative stress. We found that the mean value of 5-OHmdU was significantly decreased after three weeks of soy supplementation.[14] These results have demonstrated that isoflavone supplementation is very effective in reducing the level of 5-OhmdU, decreasing oxidative damage, and inhibition of NF-κB activation in humans *in vivo*, providing strong evidence that soy isoflavone functions as an antioxidant and that these effects of isoflavone may be responsible for its chemopreventive activity.

4.1.3. *The effects of soy isoflavone genistein on cancer cells*

The effects of isoflavone genistein on cancer cells have been widely studied in various cancer cells. The results from our laboratory and other investigators have revealed that genistein inhibits the growth of various cancer cells including leukemia, lymphoma, neuroblastoma, breast, prostate, lung, gastric, head, and neck cancer cells.[13,58–65] We and other investigators have

also found that genistein induces apoptosis with modulation of expression of genes related to apoptotic processes.[49,61,63–65] Genistein has been shown to regulate the molecules in cell signaling pathways including Akt, NF-κB, MAPK, p53, AR, and ER pathways.[48,62] By microarray and reverse transcriptase-polymerase chain reaction analysis, we have also found that genistein regulates the expression of genes that are critically involved in the control of cell growth, cell cycle, apoptosis, cell signaling transduction, angiogenesis, tumor cell invasion, and metastasis,[66,67] suggesting its pleiotropic effects on cancer cells. These effects make isoflavone a promising agent against oxidative stress, some chronic diseases, and cancers.

4.2. *Indole-3-carbinol and 3,3′-diindolylmethane*

I3C is produced from naturally occurring glucosinolates contained in a wide variety of plants including members of the family Cruciferae, and particularly members of the genus *Brassica*. I3C is biologically active and it is easily converted *in vivo* to its dimeric product DIM. Under the acidic conditions of the stomach, I3C undergoes extensive and rapid self-condensation reactions to form several derivatives.[68] DIM is the major derivative and condensation product of I3C and it is also biologically active. The formation of DIM from I3C has been believed to be a likely prerequisite for I3C-induced anti-carcinogenesis. I3C and DIM have been shown to reduce oxidative stress and stimulate antioxidant response element-driven gene expression as antioxidants.[69,70] Furthermore, we and other investigators have found that I3C and DIM inhibit oncogenesis and cancer cell growth, and induce apoptosis in various cancer cells,[9,17,71–73] suggesting that I3C and DIM may serve as potent agents for prevention and/or treatment of cancers.

We have also investigated whether I3C treatment could inhibit NF-κB DNA-binding activity in prostate and breast cancer cells by EMSA.[17,73] Cancer cells were treated with 60 or 100 μmol/l I3C for 48 h or with 20 μg/l TNF-α for 10 min. Nuclear proteins were harvested from samples, incubated in DNA-binding buffer with ^{32}P labeled NF-κB consensus oligonucleotide, and subjected to 8% non-denatured polyacrymide gel. After drying

the gel, autoradiography of the gel showed that TNF-α treatment stimulated NF-κB activation as expected; however, I3C significantly inhibited NF-κB DNA-binding activity in prostate and breast cancer cells, corresponding with the inhibition of cell proliferation and the induction of apoptosis by I3C in prostate and breast cancer cells.[17,73] These results suggest that inhibition of NF-κB activity by I3C may reduce the oxidative stress induced by ROS or TNF-α.

4.3. *Curcumin*

Curcumin is a compound from *Curcuma longa* (tumeric). *C. longa* is a plant widely cultivated in tropical regions of Asia and Central America. Turmeric extract from the rhizomes, commonly called curcuminoids, is mainly composed of curcumin. Curcumin has recently received considerable attention due to its pronounced anti-inflammatory, anti-oxidative, immunomodulating, anti-atherogenic, and anti-carcinogenic activities.[7,74–76]

Curcumin is a potent scavenger of oxygen free radicals such as hydroxyl radical and nitrogen dioxide radical.[77] It has been reported that curcumin inhibits lipid peroxidation in rat brain, liver, and lens, suggesting its antioxidant properties.[78–80] Chuang *et al.*[81] have shown that curcumin inhibits diethylnitrosamine-induced liver inflammation and activation of NF-κB in rats. Curcumin can also protect against inflammation-related changes. Administration of curcumin decreases the level of the prostanoids in alcohol toxicity model, suggesting its protective effects against inflammation.[82] It has been reported that curcumin inhibited IKK, suppressed both constitutive and inducible NF-κB activation, and potentiated TNF-induced apoptosis.[83] Curcumin also showed strong antioxidant and anticancer properties through regulating the expression of genes that require the activation of activator protein 1 and NF-κB.[84] It has been known that curcumin inhibits the growth of cancer cells, induces apoptosis, reduces cell survival signal protein Akt, and regulates the expression of genes related to anti-invasion.[15,85–87] In addition, NF-κB has been implicated in the development of drug resistance in cancer cells. Curcumin has been found to significantly inhibit chemotherapeutic agent doxorubicin-induced NF-κB activation,[88] suggesting its effect on reducing drug resistance and sensitizing cancer cell to chemotherapeutic agents.

4.4. *Epigallocatechin-3-gallate*

Consumption of green tea has been associated with human health including the prevention of cancer and heart disease. Green tea and its constituents have been studied both *in vitro* and *in vivo*. Green tea contains several catechins including epicatechin, epigallocatechin, epicatechin-3-gallate, and epigallocatechin-3-gallate (EGCG). However, EGCG has been believed to be the most potent for inhibition of oncogenesis and reduction of oxidative stress among these catechins.[24,89]

EGCG has been shown to have strong antioxidant activity. It has been reported that EGCG treatment resulted in a significant dose- and time-dependent inhibition of activation and translocation of NF-κB to the nucleus by suppressing the degradation of IκBα in the cytoplasm.[90,91] EGCG has also been shown to inhibit activation of IKK and phosphorylation of IκBα, corresponding with the inhibition of activation of NF-κB.[92,93] There are growing evidences showing that EGCG inhibits the proliferation of various cancer cells and induces apoptotic processes in cancer cells,[24,89] suggesting its inhibitory effects on cancers. It has been found that EGCG had a concurrent effect on two important transcription factors, such as p53 (stabilization of p53) and NF-κB (negative regulation of NF-κB activity), and also caused a change in the ratio of Bax/Bcl-2 in a manner that favors apoptosis.[94] Moreover, EGCG has been found to reduce the levels of matrix metalloproteinases, suppress angiogenesis, and inhibit invasion and metastasis.[95,96] In addition, EGCG also prevents oxidative modification of low density lipoproteins in human and the development of atherosclerosis in apoprotein E-deficient mice,[97,98] suggesting that EGCG may reduce the risk of cardiovascular diseases.

4.5. *Resveratrol*

Resveratrol (3,5,4′-trihydroxystilbene) is a phytoalexin present in a wide variety of plant species including grapes, mulberries, and peanuts. Relatively high quantities of resveratrol are found in grapes. The concentration of resveratrol in red wine and grape juice is in the range of 0.05–10 mg/l, depending on grape cultivar, geographical origin, and process methodology.[10] Resveratrol has been shown to have beneficial effects

on the reduction of oxidative stress and the prevention of heart diseases, degenerative diseases, and cancers.[10,99,100]

Resveratrol has been reported to modulate lipoprotein metabolism and to inhibit platelet aggregation and coagulation,[101] suggesting its preventive effects on cardiovascular diseases. It has been found that resveratrol reduces DNA damage and formation of A2E-epoxidation, which is implicated in the degenerative disease.[102] Moderate wine consumption has been associated with decreased odds of developing age-related degenerations.[103] Experimental studies have shown that resveratrol inhibits the growth of various cancer cells and induces apoptotic cell death.[104–107] The induction of apoptosis by resveratrol has been believed to be mediated through p53-depenedent, Fas, MAPK, or ceramide signaling pathway.[104–107] Resveratrol also shows their inhibitory effects on the activity of NF-κB,[18] suggesting its role as antioxidant contributing to cancer prevention and/or treatment.

4.6. *Lycopene*

Tomatoes are rich in lycopene, which is the pigment principally responsible for the deep-red color of tomato and its products. Tomato products including ketchup, tomato juice, and pizza sauce are the richest sources of lycopene in the US diet. The consumption of tomatoes and tomato products containing lycopene have been shown to be associated with decreased risk of chronic diseases such as cardiovascular diseases and cancers.[11]

Lycopene is a potent antioxidant. It has been found that lycopene, as a biologically occurring carotenoid, exhibits high physical quenching rate constant with singlet oxygen, suggesting its high activity as antioxidant.[108] Sesso *et al.*[109] have found that higher plasma lycopene concentrations are associated with a lower risk of cardiovascular diseases in women. The prevention of lipid peroxidation by lycopene may be one of the reasons that lycopene reduces the risk of atherosclerosis and cardiovascular diseases.[110] In addition to the effect on cardiovascular disease, lycopene also shows beneficial effects on cancer prevention and treatment. Giovannucci *et al.*[111] have reported that frequent consumption of tomato products is associated with a lower risk of prostate cancer. The inverse associations between plasma lycopene and prostate cancer have also been reported.[112] Experimental studies also show that lycopene inhibits cell growth in breast, prostate, and

endometrial cancer cells with regulation of cell cycle-related genes.[113,114] Clinical trial have revealed that lycopene supplements reduce tumor size and PSA level in localized prostate cancers,[115] suggesting its promising effects on prostate cancer treatment.

4.7. *Vitamins and others*

Vitamin E (α-tocopherol) is a lipid-soluble antioxidant distributed in green leaf vegetables, nuts, seeds, sunflower, and plant oils. Plant oils are the main dietary source of vitamin E. Vitamin E exerts potent antioxidant effect. It has been reported that vitamin E inhibits NF-κB activation and NF-κB-dependent transcription, and induces differentiation through reduction of NF-κB,[19,116,117] suggesting that vitamin E may exert its antioxidant effect through modulation of NF-κB. Vitamin E supplement has been associated with decreased risk of degenerative disease and cardiovascular disease.[118,119] Vitamin E also shows its inhibitory effects on carcinogenesis.[120] Vitamin E and its deriver have been known to inhibit cancer cell growth via modulating cell cycle regulatory and apoptotic machineries,[121,122] suggesting their inhibitory effects on cancers.

Vitamin C is a water-soluble antioxidant. The sources of vitamin C are fruits and vegetables, particularly orange, strawberry, citrus, kiwi, Brussels sprouts, and cauliflower.[5] It has been reported that vitamin C inhibits NF-κB activation by the inhibition of IκBα phosphorylation or the activation of p38 mitogen-activated protein kinase.[20,123]

In addition to vitamin E and C, vitamin A, ginseng, ubiquinone, ginkgo, and docosahexaenoic acid have also been known as antioxidants.[22] They may have some beneficial effects on human health, particularly in chronic diseases including cancers.

5. Conclusions

Oxidative stress has been linked to aging, some chronic diseases, and carcinogenesis. NF-κB plays important roles in oxidative stress and carcinogenesis. Therefore, targeting NF-κB may be a novel and important preventive or therapeutic strategy against some chronic diseases and

cancers. The plant-derived components (isoflavones, curcumin, EGCG, I3C, resveratrol, lycopene, vitamin E, vitamin C, etc.) have been found to reduce oxidative stress and inhibit NF-κB activation. They may have inhibitory effects on atherosclerosis, neurodegeneration, oncogenesis, cancer cell growth, and progression. These effects make them strong candidates as chemopreventive or therapeutic agents against cardiovascular diseases, degenerative diseases, and cancers.

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References

1. Finkel T, Holbrook NJ. *Nature* 239 (2000).
2. Davies KJ. *Biochem. Soc. Symp.* 1 (1995).
3. Beal MF. *Free Radic. Biol. Med.* 797 (2002).
4. Galaris D, Evangelou A. *Crit. Rev. Oncol. Hematol.* 93 (2002).
5. Benzie IF. *Comp. Biochem. Physiol. A* 113 (2003).
6. Ruiz-Larrea MB, Mohan AR, Paganga G, Miller NJ, Bolwell GP, Rice-Evans CA. *Free Radic. Res.* 63 (1997).
7. Miquel J, Bernd A, Sempere JM, Diaz-Alperi J, Ramirez A. *Arch. Gerontol. Geriatr.* 37 (2002).
8. Rietveld A, Wiseman S. *J. Nutr.* 3285S (2003).
9. Chung FL, Morse MA, Eklind KI, Xu Y. *Ann. N.Y. Acad. Sci.* 186 (1993).
10. Fremont L. *Life Sci.* 663 (2000).
11. Heber D, Lu QY. *Exp. Biol. Med. (Maywood)* 920 (2002).
12. Urso ML, Clarkson PM. *Toxicology* 41 (2003).
13. Davis JN, Kucuk O, Sarkar FH. *Nutr. Cancer* 167 (1999).
14. Davis JN, Kucuk O, Djuric Z, Sarkar FH. *Free Radic. Biol. Med.* 1293 (2001).
15. Aggarwal BB, Kumar A, Bharti AC. *Anticancer Res.* 363 (2003).
16. Gupta S, Hastak K, Afaq F, Ahmad N, Mukhtar H. *Oncogene* 2507 (2004).

17. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH. *Oncogene* 2927 (2001).
18. Estrov Z, Shishodia S, Faderl S, Harris D, Van Q, Kantarjian HM, Talpaz M, Aggarwal BB. *Blood* 987 (2003).
19. Calfee-Mason KG, Spear BT, Glauert HP. *J. Nutr.* 3178 (2002).
20. Carcamo JM, Pedraza A, Borquez-Ojeda O, Golde DW. *Biochemistry* 12995 (2002).
21. Vinson JA, Teufel K, Wu N. *J. Agric. Food. Chem.* 3661 (2004).
22. Grundman M, Grundman M, Delaney P. *Proc. Nutr. Soc.* 191 (2002).
23. Fleischauer AT, Simonsen N, Arab L. *Nutr. Cancer* 15 (2003).
24. Lambert JD, Yang CS. *J. Nutr.* 3262S (2003).
25. Makarov SS. *Mol. Med. Today* 441 (2000).
26. Karin M, Cao Y, Greten FR, Li ZW. *Nat. Rev. Cancer* 301 (2002).
27. Owuor ED, Kong AN. *Biochem. Pharmacol.* 765 (2002).
28. Dudek EJ, Shang F, Taylor A. *Free Radic. Biol. Med.* 651 (2001).
29. Toledano MB, Leonard WJ. *Proc. Natl. Acad. Sci. USA* 4328 (1991).
30. Lenardo MJ, Baltimore D. *Cell* 227 (1989).
31. Ghosh G, van Duyne G, Ghosh S, Sigler PB. *Nature* 303 (1995).
32. Muller CW, Rey FA, Harrison SC. *Nat. Struct. Biol.* 224 (1996).
33. Israel A. *Nature* 596 (2003).
34. Chen ZJ, Parent L, Maniatis T. *Cell* 853 (1996).
35. Karin M, Delhase M. *Semin. Immunol.* 85 (2000).
36. Chen F, Castranova V, Shi X. *Am. J. Pathol.* 387 (2001).
37. Barkett M, Gilmore TD. *Oncogene* 6910 (1999).
38. Beg AA, Baltimore D. *Science* 782 (1996).
39. Beg AA, Sha WC, Bronson RT, Ghosh S, Baltimore D. *Nature* 167 (1995).
40. Nichols TC, Fischer TH, Deliargyris EN, Baldwin AS Jr. *Ann. Periodontol.* 20 (2001).
41. Mattson MP, Camandola S. *J. Clin. Invest.* 247 (2001).
42. Yamamoto Y, Gaynor RB. *J. Clin. Invest.* 135 (2001).
43. Bharti AC, Aggarwal BB. *Biochem. Pharmacol.* 883 (2002).
44. Orlowski RZ, Baldwin AS. *Trends Mol. Med.* 385 (2002).
45. Aggarwal BB. *Biochem. Pharmacol.* 1033 (2000).
46. Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, Royer HD, Grinstein E, Greiner A, Scheidereit C, Dorken B. *J. Clin. Invest.* 2961 (1997).
47. Hideshima T, Chauhan D, Richardson P, Mitsiades C, Mitsiades N, Hayashi T, Munshi N, Dang L, Castro A, Palombella V, Adams J, Anderson KC. *J. Biol. Chem.* 16639 (2002).

48. Li Y, Sarkar FH. *Clin. Cancer Res.* 2369 (2002).
49. Zhou Y, Lee AS. *J. Natl. Cancer Inst.* 381 (1998).
50. Kameoka S, Leavitt P, Chang C, Kuo SM. *Cancer Lett.* 161 (1999).
51. Sierens J, Hartley JA, Campbell MJ, Leathem AJ, Woodside JV. *Teratog. Carcinog. Mutagen.* 227 (2002).
52. Ferretti G, Bacchetti T, Menanno F, Curatola G. *Atherosclerosis* 55 (2004).
53. Jiang F, Jones GT, Husband AJ, Dusting GJ. *J. Vasc. Res.* 276 (2003).
54. Kawakami Y, Tsurugasaki W, Yoshida Y, Igarashi Y, Nakamura S, Osada K. *J. Agric. Food Chem.* 1764 (2004).
55. Wei H, Wei L, Frenkel K, Bowen R, Barnes S. *Nutr. Cancer* 1 (1993).
56. Baxa DM, Yoshimura FK. *Biochem. Pharmacol.* 1009 (2003).
57. Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E, Jacquot J. *Am. J. Pathol.* 473 (1999).
58. Constantinou A, Kiguchi K, Huberman E. *Cancer Res.* 2618 (1990).
59. Buckley AR, Buckley DJ, Gout PW, Liang H, Rao YP, Blake MJ. *Mol. Cell Endocrinol.* 17 (1993).
60. Zhou HB, Chen JJ, Wang WX, Cai JT, Du Q. *World J. Gastroenterol.* 1822 (2004).
61. Alhasan SA, Pietrasczkiwicz H, Alonso MD, Ensley J, Sarkar FH. *Nutr. Cancer* 12 (1999).
62. Gong L, Li Y, Nedeljkovic-Kurepa A, Sarkar FH. *Oncogene* 4702 (2003).
63. Li Y, Bhuiyan M, Sarkar FH. *Int. J. Oncol.* 525 (1999).
64. Li Y, Upadhyay S, Bhuiyan M, Sarkar FH. *Oncogene* 3166 (1999).
65. Lian F, Bhuiyan M, Li YW, Wall N, Kraut M, Sarkar FH. *Nutr. Cancer* 184 (1998).
66. Li Y, Sarkar FH. *J. Nutr.* 3623 (2002).
67. Li Y, Sarkar FH. *Cancer Lett.* 157 (2002).
68. Verhoeven DT, Verhagen H, Goldbohm RA, van den Brandt PA, van Poppel G. *Chem. Biol. Interact.* 79 (1997).
69. Nho CW, Jeffery E. *Toxicol. Appl. Pharmacol.* 40 (2004).
70. Benabadji SH, Wen R, Zheng JB, Dong XC, Yuan SG. *Acta Pharmacol. Sin.* 666 (2004).
71. Sarkar FH, Rahman KM, Li Y. *J. Nutr.* 2434S (2003).
72. Firestone GL, Bjeldanes LF. *J. Nutr.* 2448S (2003).
73. Rahman KW, Li Y, Sarkar FH. *Nutr. Cancer* 84 (2004).
74. Banerjee M, Tripathi LM, Srivastava VM, Puri A, Shukla R. *Immunopharmacol. Immunotoxicol.* 213 (2003).
75. Ramirez-Tortosa MC, Mesa MD, Aguilera MC, Quiles JL, Baro L, Ramirez-Tortosa CL, Martinez-Victoria E, Gil A. *Atherosclerosis* 371 (1999).

76. Rao CV, Rivenson A, Simi B, Reddy BS. *Cancer Res.* 259 (1995).
77. Tonnesen HH, Greenhill JV. *Int. J. Pharm.* 79 (1992).
78. Reddy AC, Lokesh BR. *Toxicology* 39 (1996).
79. Awasthi S, Srivatava SK, Piper JT, Singhal SS, Chaubey M, Awasthi YC. *Am. J. Clin. Nutr.* 761 (1996).
80. Shukla PK, Khanna VK, Khan MY, Srimal RC. *Hum. Exp. Toxicol.* 653 (2003).
81. Chuang SE, Cheng AL, Lin JK, Kuo ML. *Food Chem. Toxicol.* 991 (2000).
82. Jayadeep VR, Arun OS, Sudhakaran PR, Menon VP. *J. Nutr. Biochem.* 509 (2000).
83. Bharti AC, Donato N, Singh S, Aggarwal BB. *Blood* 1053 (2003).
84. Duvoix A, Morceau F, Delhalle S, Schmitz M, Schnekenburger M, Galteau MM, Dicato M, Diederich M. *Biochem. Pharmacol.* 1475 (2003).
85. Radhakrishna PG, Srivastava AS, Hassanein TI, Chauhan DP, Carrier E. *Cancer Lett.* 163 (2004).
86. Chen HW, Yu SL, Chen JJ, Li HN, Lin YC, Yao PL, Chou HY, Chien CT, Chen WJ, Lee YT, Yang PC. *Mol. Pharmacol.* 99 (2004).
87. Chaudhary LR, Hruska KA. *J. Cell Biochem.* 1 (2003).
88. Chuang SE, Yeh PY, Lu YS, Lai GM, Liao CM, Gao M, Cheng AL. *Biochem. Pharmacol.* 1709 (2002).
89. Mukhtar H, Ahmad N. *Toxicol. Sci.* 111 (1999).
90. Afaq F, Adhami VM, Ahmad N, Mukhtar H. *Oncogene* 1035 (2003).
91. Ahmad N, Gupta S, Mukhtar H. *Arch. Biochem. Biophys.* 338 (2000).
92. Chen PC, Wheeler DS, Malhotra V, Odoms K, Denenberg AG, Wong HR. *Inflammation* 233 (2002).
93. Yang F, Oz HS, Barve S, de Villiers WJ, McClain CJ, Varilek GW. *Mol. Pharmacol.* 528 (2001).
94. Hastak K, Gupta S, Ahmad N, Agarwal MK, Agarwal ML, Mukhtar H. *Oncogene* 4851 (2003).
95. Kim HS, Kim MH, Jeong M, Hwang YS, Lim SH, Shin BA, Ahn BW, Jung YD. *Anticancer Res.* 747 (2004).
96. Jung YD, Ellis LM. *Int. J. Exp. Pathol.* 309 (2001).
97. Miura Y, Chiba T, Tomita I, Koizumi H, Miura S, Umegaki K, Hara Y, Ikeda M, Tomita T. *J. Nutr.* 27 (2001).
98. Miura Y, Chiba T, Miura S, Tomita I, Umegaki K, Ikeda M, Tomita T. 216 (2000).
99. Dong Z. *Mutat. Res.* 145 (2003).
100. Ignatowicz E, Baer-Dubowska W. *Pol. J. Pharmacol.* 557 (2001).

101. Olas B, Wachowicz B, Saluk-Juszczak J, Zielinski T. *Thromb. Res.* 141 (2002).
102. Sparrow JR, Vollmer-Snarr HR, Zhou J, Jang YP, Jockusch S, Itagaki Y, Nakanishi K. *J. Biol. Chem.* 18207 (2003).
103. Obisesan TO, Hirsch R, Kosoko O, Carlson L, Parrott M. *J. Am. Geriatr. Soc.* 1 (1998).
104. Laux MT, Aregullin M, Berry JP, Flanders JA, Rodriguez E. *J. Altern. Complement. Med.* 235 (2004).
105. Scarlatti F, Sala G, Somenzi G, Signorelli P, Sacchi N, Ghidoni R. *FASEB J.* 2339 (2003).
106. Delmas D, Rebe C, Lacour S, Filomenko R, Athias A, Gambert P, Cherkaoui-Malki M, Jannin B, Dubrez-Daloz L, Latruffe N, Solary E. *J. Biol. Chem.* 41482 (2003).
107. Shih A, Davis FB, Lin HY, Davis PJ. *J. Clin. Endocrinol. Metab.* 1223 (2002).
108. Di Mascio P, Kaiser S, Sies H. *Arch. Biochem. Biophys.* 532 (1989).
109. Sesso HD, Buring JE, Norkus EP, Gaziano JM. *Am. J. Clin. Nutr.* 47 (2004).
110. Visioli F, Riso P, Grande S, Galli C, Porrini M. *Eur. J. Nutr.* 201 (2003).
111. Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. *J. Natl. Cancer Inst.* 391 (2002).
112. Lu QY, Hung JC, Heber D, Go VL, Reuter VE, Cordon-Cardo C, Scher HI, Marshall JR, Zhang ZF. *Cancer Epidemiol. Biomarkers Prev.* 749 (2001).
113. Nahum A, Hirsch K, Danilenko M, Watts CK, Prall OW, Levy J, Sharoni Y. *Oncogene* 3428 (2001).
114. Kim L, Rao AV, Rao LG. *J. Med. Food* 181 (2002).
115. Kucuk O, Sarkar FH, Djuric Z, Sakr W, Pollak MN, Khachik F, Banerjee M, Bertram JS, Wood DP Jr. *Exp. Biol. Med. (Maywood)* 881 (2002).
116. Hirano F, Tanaka H, Miura T, Hirano Y, Okamoto K, Makino Y, Makino I. *Immunopharmacology* 31 (1998).
117. Sokoloski JA, Hodnick WF, Mayne ST, Cinquina C, Kim CS, Sartorelli AC. *Leukemia* 1546 (1997).
118. Dutta A, Dutta SK. *J. Am. Coll. Nutr.* 258 (2003).
119. Fariss MW, Zhang JG. *Toxicology* 129 (2003).
120. Omer B, Akkose A, Kolanci C, Oner P, Ozden I, Tuzlali S. *J. Natl. Cancer Inst.* 972 (1997).
121. Ni J, Chen M, Zhang Y, Li R, Huang J, Yeh S. *Biochem. Biophys. Res. Commun.* 357 (2003).
122. Gunawardena K, Murray DK, Meikle AW. *Prostate* 287 (2000).
123. Bowie AG, O'Neill LA. *J. Immunol.* 7180 (2000).

DOWNREGULATION OF ANDROGEN RECEPTOR BY DIM CONTRIBUTES TO CELL GROWTH INHIBITION AND INDUCTION OF APOPTOSIS IN BOTH HOEMONE SENSITIVE LNCAP AND INSENSITIVE C4-2B PROSTATE CANCER CELLS *

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Prostate caner, for most part, is a treatable disease and hormone ablation therapy is generally effective. However, almost all patients treated with hormone ablation therapy eventually give rise to hormone-refractory prostate cancer (HRPC) to which there is no curative therapy. Previous studies from our laboratory and others have shown anti-proliferative and pro-apoptotic effects of 3,3'-diindolylmethane (DIM) in prostate cancer cells. However, the molecular mechanism of action of DIM has not been investigated in AR positive hormone responsive and non-responsive prostate cancer cells. Therefore, we investigated the effects of B-DIM, a formulated DIM with greater bioavailability, on AR, Akt, and NF-κB signaling in hormone-sensitive LNCaP (AR+) and hormone-insensitive C4-2B (AR+) prostate cancer cells. We found that B-DIM significantly inhibited cell growth and induced apoptosis in both the cell lines. By Akt gene transfection, RT-PCR, Western Blot analysis, and EMSA, we found a potential crosstalk between Akt, NF-κB, and AR. Importantly, B-DIM significantly inhibited Akt activation, NF-κB DNA binding activity, and the expressions of AR and PSA, suggesting that B-DIM could interrupt the crosstalk. Moreover, our confocal image study revealed that B-DIM inhibited AR nuclear translocation, leading to the down-regulation of AR target genes. These results suggest that the inhibition of cell growth and the induction of apoptosis by B-DIM are partly mediated through down-regulation of AR, Akt and NF-κB signaling. These observations provide a scientific rationale for devising novel therapeutic approaches for the treatment of hormone sensitive but most importantly hormone-refractory prostate cancer by using B-DIM alone or in combination with other therapeutics.

Prostate cancer is the most frequently diagnosed cancer and the leading cause of cancer death in men in the United States with an estimated 234,460 new cases and 27,350 deaths in 2006 (1). Despite an initial efficacy of androgen-deprivation therapy, most patients with advanced prostate cancer eventually develop resistance to this therapy and progress to hormone-refractory prostate cancer (HRPC) for which there is no curative therapy (2).

Chemotherapy for prostate cancer has been used for a number of years (3), however, only limited improvement in survival was recently observed in hormone-refractory prostate cancers when treated with docetaxel-based combination treatment (4). Therefore, novel targeted therapeutic approaches must be developed for the treatment of HRPC.

During the progression of prostate cancers from androgen sensitive status to androgen independent stage, prostate cancer cells still contain androgen receptor (AR), suggesting that AR signaling plays a critical role in the development and progression of prostate cancer (5). AR is a member of the steroid receptor superfamily and a nuclear transcription factor. Upon binding to AR, androgen activates AR which, in turn, interacts with androgen response elements (AREs) in the promoter of target genes including prostate-specific antigen (PSA), regulating the transcription of target genes. PSA is a clinically important marker used to monitor diagnosis, treatment response, prognosis and progression in patients with prostate cancer (6). In addition to androgen, the activity of AR may be modified by the molecules in other cell signaling pathways. It has been reported that Akt and NF-κB regulate AR signaling pathway by phosphorylation of AR or transcriptional regulation of AR (7, 8). Akt specifically binds to AR and phosphorylates serines 213 and 791, thereby activating AR (7). Blocking Akt pathway by a dominant-negative Akt or an inhibitor of Akt abrogates the HER-2/neu-induced AR signaling (7). These results suggest that Akt is an activator of AR required for androgen-independent survival and growth of prostate cancer cells mediated by HER-2/neu signaling. It has been known that there are NF-κB binding sites in the promoter of AR (8), suggesting that NF-κB may regulate the expression of AR. The activation of Akt and NF-κB has been involved in the progression of prostate cancer from androgen dependence to independence (9, 10). In hormone-refractory prostate cancer, promiscuous function of AR together with the activation of Akt and NF-κB pathways promotes cancer cells to become resistant to androgen-deprivation therapy (9-12). In addition, androgen is also known to produce oxidative stress resulting in the production of reactive oxygen species (ROS) that, in turn, activate NF-κB and contribute to the induction of tumor cell proliferation (13). Therefore, AR, Akt, and NF-κB could be potential targets for the treatment of prostate cancer, especially HRPC.

3,3'-diindilylmethane (DIM), an *in vivo* dimeric product of indole-3-carbinol (I3C), exhibits potent anti-proliferative activities against various cancers including prostate cancer (14-16). We have reported that I3C significantly induced apoptosis and inhibited NF-κB and Akt activation in breast and prostate cancer cells, suggesting that I3C could be chemopreventive and /or therapeutic agents for breast and prostate cancers (17-19). In addition, DIM has been shown to be an androgen antagonist (20), suggesting that the growth inhibitory effects of DIM on prostate cancer could be due to inactivation of multiple signaling pathways including AR signaling. To enhance the effects of DIM, Anderton et al reported a formulated DIM (B-DIM from BioResponse) which showed approximately 50% higher bioavailability (21). However, no studies have been reported to date to elucidate the effect and molecular mechanisms of action of B-DIM on prostate cancer cells, especially on HRPC cells. To explore such mechanisms, in this study, we investigated the effects of B-DIM on a paired androgen-sensitive LNCaP and androgen-insensitive C4-2B (derived from LNCaP cells) prostate cancer cell lines. Here we report that B-DIM is a potent agent for

inducing growth inhibition and apoptotic cell death of both androgen-sensitive LNCaP and androgen-insensitive C4-2B prostate cancer cells. This effect of B-DIM was partly mediated through the regulation of AR, Akt, and NF-κB signaling pathways.

Experimental Procedures

Cell lines, Reagents, and Antibodies - Human prostate cancer cell lines including LNCaP, C4-2B, PC-3, and PC-3 stably transfected with AR were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a 5% CO_2 atmosphere at 37^{o}C. B-DIM was generously provided by Dr. Michael Zeligs (BioResponse, Boulder, CO) and was dissolved in DMSO to make a 50 mM stock solution. DHT (Sigma, St. Louis, MO) was dissolved in ethanol to make 100 μM stock solutions. Anti-AR (Santa Cruz, Santa Cruz, CA), anti-PSA (Labvision, Fremont, CA), anti-Akt (Santa Cruz), anti-pAkt Ser473 (Cell Signaling, Danvers, MA), anti-NF-κB p65 (Uptate, Charlottesville, VA), and anti-β-actin (Sigma) primary antibodies were used for Western Blot analysis or confocal microscopic study.

Cell Growth Inhibition Studies by MTT Assay - Human LNCaP and C4-2B prostate cancer cells were seeded in 96-well plates. After 24 hours, the cells were treated with 0.1, 1, 10, 25, and 50 μM B-DIM for 48-72 hours. Control cells were treated with 0.1% DMSA (vehicle control). After treatment, the cells were incubated with MTT (0.5 mg/ml, Sigma) in medium at 37°C for 2 hours and then with isopropanol at room temperature for 1 hour. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan, Durham, NC) at 595 nm.

Histone/DNA ELISA for Detection of Apoptosis - The Cell Death Detection ELISA Kit (Roche, Palo Alto, CA) was used to detect apoptosis in prostate cancer cells treated with B-DIM according to the manufacturer's protocol. Briefly, the cytoplasmic histone/DNA fragments from LNCaP and C4-2B cells treated with 0, 10, and 25 μM B-DIM for 24, 48, and 72 hours were extracted and incubated in microtiter plate modules coated with anti-histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized histone/DNA fragments followed by color development with ABTS substrate for peroxidase. The spectrophotometric absorbance of the samples was determined by using Ultra Multifunctional Microplate Reader (Tecan) at 405 nm.

Western Blot Analysis - LNCaP, C4-2B, PC-3, and PC-3 cells stably transfected with AR were cultured in RPMI-1640 with 10% FBS or 10% dextran-coated charcoal-stripped FBS (DCC-FBS). Cells were then treated with B-DIM at various concentrations for different time periods followed by treatment with and without DHT (0.1, 1 nM) for 2 hours. After treatment, cells were lysed and protein concentration was then measured using BCA protein assay (Pierce, Rockford, IL). The proteins were subjected to SDS-PAGE, and electrophoretically transferred to nitrocellulose membrane. The membranes were incubated with various primary antibodies, and subsequently incubated with secondary antibody conjugated with peroxidase. The signal was then detected using the chemiluminescent detection system (PIERCE).

Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis – LNCaP, C4-2B, and AR stably transfected PC-3 cells were treated as

described above. Total RNA was extracted using Trizol (Invitrogen) and purified by using RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (2 μg) from each sample was subjected to reverse transcription using the SuperScript First-Strand cDNA Synthesis Kit (Invitrogen) and the cDNAs were subjected to real-time PCR analysis for AR and PSA expression. Real-time PCR reactions were carried out in SmartCycler II (Cepheid, Sunnyvale, CA). The primers for AR were as follows: 5'-AGCCATTGAGCCAGGTGTAG-3' and 5'-CGTGTAAGTTGCGGAAGCC-3'. The primers for PSA were as follows: 5'-GTGGGTCCCGGTTGTCT-3' and 5'-AGCCCAGCTCCCTGTCT-3'. PCR amplification efficiency and linearity for each gene including targeted and control genes were tested. Data were analyzed according to the comparative cycle threshold (Ct) method and were normalized by β-actin or GAPDH expression in each sample. Melting curves for each PCR reaction were generated to ensure the purity of the amplification product.

NF-κB DNA Binding Activity Measurement - LNCaP and C4-2B cells were treated as described above. Nuclear extracts were prepared according to the method described by Chaturvedi et al (22) and the protein concentration was measured. Nuclear protein was then subjected to electrophoretic mobility shift assay (EMSA). EMSA was performed by incubating 4μg of nuclear proteins with IRDye^{TM}-700 labeled NF-κB oligonucleotide and 2μg of poly (dI-dC) for 30 minutes at room temperature in the dark. The DNA-protein complex formed was separated from free oligonucleotide on an 8% native polyacrylamide gel followed by scanning with the Odyssey Imaging System (LI-COR, Lincoln, NB).

Immunofluorescence Staining and Confocal Imaging - LNCaP and C4-2B cells were plated on cover slips in each well of 6-well plate containing 10% DCC-FBS. Cells were then treated with B-DIM (10, 25 μM) for different time periods followed by incubation with and without DHT (0.1, 1 nM) for 2 hours. Cells were then fixed with 10% formalin for 10 minutes. Then, cover slips were rinsed with PBS, treated with 0.2% BSA in PBS for 45 min and with 0.5% Triton X100 in PBS for 10 minutes, and incubated with Anti-AR monoclonal antibody (1:50, Santa Cruz) at 37°C for 2.5 hours in PBS with 0.5% Triton X100. After washing with PBS, the cells were incubated with FITC-conjugated anti-mouse antibody (1:100, Molecular probes, Eugene, OR) along with 0.1 μg/ml DAPI (Sigma) at 37°C for 1 hour and washed with PBS. Cell images were captured on a Zeiss 310 laser scanning inverted confocal microscope system, using 63 X 1.2 objective and 488/364 nm laser wavelengths to detect FITC and DAPI, respectively.

Transient Transfection with Akt cDNAs and/or Reporter Constructs – pLNCX-Akt (wild type Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control empty vector) were generously provided by Dr. Sellers (Dana-Farber Cancer Institute, Boston, MA). NF-κB-Luc (Stratagene, La Jolla, CA) contains six repeated copies of the NF-κB DNA-binding site and a luciferase reporter gene. pSV-β-gal reporter vector (Promega, Madison, WI) transfection was used for normalization of transfection efficiency. The pLNCX-Akt, pLNCX-Myr-Akt, pLNCX-Akt-K179M, or pLNCX was transiently co-transfected with NF-κB-Luc and pSV-β-gal into LNCaP and C4-2B cells using ExGen 500 (Fermentas, Hanover, MD). After 5 hours, the transfected cells were washed and incubated

with complete RPMI-1640 medium overnight followed by treatment with 50 μM B-DIM for 48 hours. Subsequently, the luciferase activities in the samples were measured by Steady-Glo™ Luciferase Assay System (Promega) and ULTRA Multifunctional Microplate Reader (TECAN). β-galactosidase activities were measured using β-galactosidase Enzyme Assay System (Promega). The nuclear proteins from transfected cells were also extracted and subjected to measurement of NF-κB DNA-binding activity using EMSA method as described above. The protein expressions of AR, PSA, Akt, p-Akt(Ser473), and NF-κB in transfected LNCaP and C4-2B cells treated with or without B-DIM were measured by Western Blot analysis.

LNCaP and C4-2B cells were also transiently co-transfected with PSA-Luc promoter construct containing ARE and pSV-β-gal vector by ExGen 500 (Fermentas). PSA-Luc construct was generously provided by Dr. Charles Young (Mayo Clinic, Rochester, MN). After 5 hours, the transfected cells were washed and incubated with complete RPMI-1640 medium overnight followed by treatment with 10 and 25 μM B-DIM for 24 and 48 hours. Subsequently, the luciferase and β-galactosidase activities in the samples were measured as described above.

RESULTS

Inhibition of Cell Growth by B-DIM - Prior to our molecular experiments, we tested the effect of androgen (DHT) on the growth of LNCaP and C4-2B cells. As expected, LNCaP cells which are known to be sensitive to androgen showed growth stimulation of 25% and 35% at 48 and 72 hours upon 1 nM DHT treatment. In contrast, C4-2B cells which are insensitive to androgen showed minimal or no growth stimulation (data not shown). Subsequently, we tested the effects of B-DIM on cell growth in prostate cancer cells by MTT assay. We found that the treatment of LNCaP and C4-2B prostate cancer cells with B-DIM resulted in a dose- and time-dependent inhibition of cell proliferation (Fig. 1A and 1B) with maximal inhibition seen at 50 μM, demonstrating a potent growth inhibitory effect of B-DIM on both androgen sensitive and insensitive prostate cancer cells. Because the inhibition of cell growth by B-DIM could be also due to the induction of apoptosis, we next tested the apoptosis inducing effects of B-DIM in prostate cancer cells.

Induction of Apoptosis by B-DIM in Prostate Cancer Cells - To investigate whether the growth inhibitory effects B-DIM is due to the induction of apoptosis, the Histone/DNA ELISA was conducted in B-DIM-treated LNCaP and C4-2B prostate cancer cells. We observed a significant induction of apoptosis in both androgen dependent and independent prostate cancer cells (Fig. 1C and 1D). This induction of apoptosis was time-dependent and directly correlated with the inhibition of cell growth, suggesting that the growth inhibitory activity of B-DIM was partly mediated through an increase in apoptotic cell death. These results are in direct agreement with those observed earlier in I3C treated breast and prostate cancer cells (17-19). Since AR and PSA play critical roles in the initiation and progression of prostate cancer, we detected the effects of B-DIM on AR and PSA expression by RT-PCR and Western Blot analysis.

Inhibition of AR and PSA Expressions in Prostate Cancer Cells – By Western Blot analysis, we found that B-DIM significantly inhibited the expression levels of AR and PSA proteins in LNCaP prostate cancer cells in both time-dependent and dose-dependent

manners (Fig. 2A). Similarly, B-DIM also showed significant time-dependent and dose-dependent inhibition of AR and PSA protein expression in C4-2B prostate cancer cells (Fig. 3A). Furthermore, B-DIM abrogated the DHT-induced up-regulation of AR and PSA proteins in both LNCaP (Fig. 2B) and C4-2B (Fig. 3B) cells.

In order to examine whether down-regulation of AR and PSA proteins by B-DIM is a transcriptional event, we examined the expression levels of both AR and PSA mRNA by real-time RT-PCR. The results showed that B-DIM significantly down-regulated the expression of both AR and PSA mRNA in the presence and absence of DHT in both LNCaP (Fig. 2C and 2D) and C4-2B cells (Fig. 3C and 3D), indicating that B-DIM could inhibit the basic and the DHT-induced AR and PSA transcriptional activities. These results are consistent with B-DIM-regulated AR and PSA expression at the protein levels, suggesting that B-DIM-regulated AR and PSA gene expressions were transcriptional events.

In order to further prove this point, we tested the effects of B-DIM on AR stably transfected PC-3 prostate cancer cells in which the expression of AR is driven by an artificial promoter. We did not observed down-regulation of AR by B-DIM in AR stably transfected PC-3 cells (Fig. 4A). We also transfected PSA-Luc vector, which contains PSA promoter with ARE and luciferase reporter gene, into LNCaP and C4-2B cells and treated the cells with B-DIM. We found that luciferase activity was significantly induced after PSA-Luc transfection and 10-25 μM B-DIM treatment for 24-48 hours significantly decreased luciferase activity in PSA-Luc transfected LNCaP and C4-2B cells (Fig. 4B and 4C). These results further demonstrated that the down-regulation of AR and PSA expression by B-DIM is a transcriptional event. Because it is reported that AR may be regulated by Akt and NF-κB (7, 8), we next tested the effects of B-DIM on Akt and NF-κB signaling.

Down-regulation of Akt by B-DIM leading to inhibition of NF-κB, AR, and PSA – By Western Blot analysis, we found that B-DIM down-regulated the protein levels of p-Akt, nuclear NF-κB, AR, and PSA in both LNCaP and C4-2B cells (Fig. 5). To further investigate the relationship between Akt, NF-κB, AR, and PSA, and the effects of B-DIM on these molecules, we co-transfected Akt cDNA and NF-κB-Luc into LNCaP and C4-2B cells. We found that p-Akt, nuclear NF-κB, AR, and PSA were up-regulated after wild type Akt and Myr Akt transfections in LNCaP and C4-2B cells. However, the up-regulations of p-Akt, nuclear NF-κB, AR, and PSA by Akt transfection were significantly abrogated in both LNCaP and C4-2B cells (Fig. 5).

By tranfection and luciferase assay, we found that luciferase activity was significantly increased after co-transfection with NF-κB-Luc and wild type Akt or Myr Akt in both LNCaP and C4-2B cells (Fig. 6A), suggesting the activation of NF-κB by Akt transfection. Moreover, B-DIM treatment significantly abrogated the up-regulation of luciferase activity caused by Akt transfection. Furthermore, we conducted EMSA to test NF-κB DNA binding activity in Akt transfected cells. The results showed that NF-κB DNA binding activity was significantly increased by wild type Akt and Myr Akt transfection and this was inhibited by B-DIM treatment (Fig. 6B), which is consistent with the data from luciferase assay. Moreover, we also found that B-DIM at 10 or 25 μM significantly inhibited NF-κB DNA binding activity in the presence and absence of DHT in both LNCaP and C4-2B cells (Fig. 6C).

Combined with the alteration in AR as observed by Akt transfection studies, our

results suggest that there could be a crosstalk between p-Akt, NF-κB, and AR, and that the inhibition of Akt activation by B-DIM could lead to the down-regulation of NF-κB, AR, and PSA. Because the function of AR in the regulation of its target genes is mainly occurred in the nucleus, we next tested the localization of AR before and after B-DIM treatment.

Inhibition of AR Nuclear Translocation by B-DIM - By Western Blot analysis, we found that B-DIM reduced AR protein level in both the cytosol and the nuclear extracts (Fig. 5). However, the AR protein level was much more down-regulated by B-DIM in nucleus than that in cytosol, suggesting that B-DIM could inhibit AR nuclear translocation. Therefore, we conducted immunofluorescent staining and confocal imaging to examine the effect of B-DIM on AR nuclear translocation in LNCaP and C4-2B prostate cancer cells. We found that both LNCaP and C4-2B cells treated with 25 μM B-DIM for 24 hours showed much less AR staining in nucleus compared to control (Fig. 7A and 7B). These results suggest that B-DIM significantly inhibited AR translocation into the nucleus and, in turn, down-regulated AR target genes including PSA, consistent with RT-PCR and Western Blot data showing down-regulation of PSA mRNA and protein levels after B-DIM treatment (Fig. 2, 3, and 5).

DISCUSSION

Androgen exerts its biological effects through binding to AR and activating AR transcriptional activity, promoting growth of prostate epithelial cells. Therefore, androgen and AR play important roles in the normal development and maintenance of prostate (5). More importantly, AR signaling also plays critical roles in the development and progression of prostate cancer. In the early stage of prostate cancer, androgen-deprivation therapy shows inhibition of cancer cell growth, suggesting that androgen still controls functions of AR signaling at this stage. However, in hormone-refractory prostate cancer (HRPC), androgen-deprivation therapy fails although AR expression is still maintained in most cases (5). Actually, 30% of HRPC show amplification and over-expression of AR gene, indicating that AR signaling pathway is malfunctioning in HRPC (23, 24). Studies on the progression of prostate cancer have indicated that an increase in AR mRNA and protein is both necessary and sufficient to convert prostate cancer from a hormone-sensitive to a hormone refractory stage (25), and that overexpressed AR linked to $p21^{waf1}$ silencing may be responsible for androgen independence and resistance to apoptosis (26). These studies suggest that over-expression of AR is an important factor for acquired resistance of prostate cancer to androgen-deprivation therapy. Therefore, AR is a key target for the treatment of both early stage of prostate cancer and HRPC and the inactivation of AR expression should be an important approach for the successful treatment of HRPC. In this study, we found that B-DIM significantly inhibited cell growth, induced apoptosis, down-regulated the expression of AR mRNA and protein, and abrogated the activation of AR by DHT in both androgen sensitive and insensitive prostate cancer cells. These results suggest that cell growth inhibition and induction of apoptosis by B-DIM is partly mediated through the down-regulation of AR. B-DIM also inhibited AR nuclear translocation and subsequent expression of PSA, one of the important makers for progression of prostate cancer. These results suggest that B-DIM could be a potent agent for the treatment of androgen sensitive but most importantly androgen insensitive prostate cancer.

Several cell signal transduction pathways have been involved in the

progression of HRPC by the interaction with AR signaling (27-29). Among them, Akt pathway is an important cell signaling pathway for the survival of prostate cancer cells (30). It is believed that increased AR activity is caused by a crosstalk between AR, PI3K/Akt, and MAPK pathways (27) although the relationship between Akt and AR remains controversial. It has been reported that Akt directly or indirectly interacts with AR through GSK3β and β-catenin, and then enhances AR transactivation, promoting the growth of prostate cancer cells (7, 28, 31). However, recent study by Yang et al showed that inhibition of PI3K/Akt pathway by LY294002 could result in the activation of FOXO3a, which could then induce AR expression to protect prostate cancer cells from apoptosis caused by the inhibition of PI3K/Akt pathway (32). Therefore, the inhibition of both Akt and AR signaling pathways may be a powerful approach to treat both androgen dependent prostate cancer and HRPC. In this study, we found that B-DIM inhibited both Akt activation and AR transactivation, suggesting that B-DIM could be a potent agent for the treatment of both androgen dependent prostate cancer and HRPC. We also found that transfection of Akt caused activation of NF-κB and increase in AR expression, suggesting that there could be a direct crosstalk between Akt, NF-κB, and AR.

It has been well known that NF-κB pathway plays important roles in the control of cell growth, differentiation, apoptosis, inflammation, stress response, and many other physiological processes in cellular signaling. NF-κB signaling pathway is also involved in the development and progression of prostate cancer. NF-κB is overexpressed in prostatic intraepithelial neoplasia and prostate adenocarcinoma (33). Constitutive activation of NF-κB has been found in androgen independent prostate cancer cells while less activity of NF-κB has been observed in androgen dependent prostate cancer cells (34, 35). Like Akt and AR, the relationship between NF-κB and AR activation remains controversial. Palvimo et al reported that elevated expression of NF-κB p65 repressed AR-mediated transactivation in a dose-dependent manner whereas NF-κB p50 did not influence AR transactivation (36). However, other investigators show that IL-4 induced NF-κB is required for AR activation (29) and that there are NF-κB binding sites in the promoter of AR (8), suggesting that the activation of NF-κB could enhance AR transactivation. Therefore, inhibition of both NF-κB and AR could be another powerful approach to treat both androgen dependent prostate cancer and HRPC. Indeed, in this study, we found that B-DIM inhibited NF-κB, AR, and PSA, resulting in the cell growth inhibition and apoptotic cell death in both androgen sensitive and insensitive prostate cancer cells. It has been reported that NF-κB activates PSA expression by direct binding to the enhancer of PSA (37). Therefore, the inhibition of PSA expression by B-DIM could be mediated through the down-regulation of both NF-κB and AR.

In HRPC, the failure of androgen-deprivation therapy is believed to be due to the AR modifications including AR mutations, AR amplification, and ligand-independent activation of AR through crosstalk with other signaling pathways (23, 24, 38, 39). Growing evidences demonstrate a critical role of AR activation by nonandrogen in the development of androgen independent prostate cancer (7, 27-29). Therefore, it is important to discover other non-androgen molecules which activate AR. By Akt transfection, we observed increased AR expression accompanied with the increased p-Akt, NF-κB, and PSA, suggesting a crosstalk between Akt, NF-κB, AR, and PSA (Fig.

7C). It has been reported that Akt regulates NF-κB activation through IKK phosphorylation (40) and that NF-κB may activate AR signaling (29). Therefore we believe that in the crosstalk, p-Akt could activate NF-κB and, in turn, activate AR which transactivate PSA expression, promoting the growth of prostate cancer cells. In addition, AR could also be activated directly by activated Akt (7) whereas PSA could be up-regulated directly by NF-κB (37). More importantly, we found that B-DIM could inhibit Akt, NF-κB, AR, and PSA in both androgen sensitive and insensitive prostate cancer cells, demonstrating its effects on the interruption of these crosstalk. Taken together, these results along with our findings on the inhibition of cell growth and the induction of apoptosis by B-DIM in both LNCaP and C4-2B prostate cancer cells, clearly suggest that B-DIM could be a promising non-toxic agent for the treatment of prostate cancer, especially hormone-refractory prostate cancer (HRPC) for which there is no curative therapy. However, further in-depth studies, including animal experiments and clinical trials, are needed to fully appreciate the value of B-DIM in the fight against prostate cancer.

REFERENCES

1. Jemal A, Siegel R, Ward E et al. Cancer Statistics, 2006. CA Cancer J Clin 2006;56**:**106-30.
2. Bracarda S, de CO, Greco C et al. Cancer of the prostate. Crit Rev Oncol Hematol 2005;56**:**379-96.
3. Denmeade SR, Isaacs JT. A history of prostate cancer treatment. Nat Rev Cancer 2002;2**:**389-96.
4. Ryan CJ, Eisenberger M. Chemotherapy for Hormone-Refractory Prostate Cancer: Now It's a Question of "When?". J Clin Oncol 2005;23**:**8242-6.
5. Heinlein CA, Chang C. Androgen Receptor in Prostate Cancer. Endocr Rev 2004;25**:**276-308.
6. Lieberman R. Evidence-based medical perspectives: the evolving role of PSA for early detection, monitoring of treatment response, and as a surrogate end point of efficacy for interventions in men with different clinical risk states for the prevention and progression of prostate cancer. Am J Ther 2004;11**:**501-6.
7. Wen Y, Hu MCT, Makino K et al. HER-2/neu Promotes Androgen-independent Survival and Growth of Prostate Cancer Cells through the Akt Pathway. Cancer Res 2000;60**:**6841-5.
8. Zhang L, Charron M, Wright WW et al. Nuclear factor-kappaB activates transcription of the androgen receptor gene in Sertoli cells isolated from testes of adult rats. Endocrinology 2004;145**:**781-9.
9. Kikuchi E, Horiguchi Y, Nakashima J et al. Suppression of hormone-refractory prostate cancer by a novel nuclear factor kappaB inhibitor in nude mice. Cancer Res 2003;63**:**107-10.
10. Murillo H, Huang H, Schmidt LJ, Smith DI, Tindall DJ. Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state. Endocrinology 2001;142**:**4795-805.

11. Pfeil K, Eder IE, Putz T et al. Long-term androgen-ablation causes increased resistance to PI3K/Akt pathway inhibition in prostate cancer cells. Prostate 2004;58**:**259-68.
12. Edwards J, Bartlett JM. The androgen receptor and signal-transduction pathways in hormone-refractory prostate cancer. Part 1: Modifications to the androgen receptor. BJU Int 2005;95**:**1320-6.
13. Ripple MO, Henry WF, Schwarze SR, Wilding G, Weindruch R. Effect of antioxidants on androgen-induced AP-1 and NF-kappaB DNA-binding activity in prostate carcinoma cells. J Natl Cancer Inst 1999;91**:**1227-32.
14. Firestone GL, Bjeldanes LF. Indole-3-carbinol and 3-3'-diindolylmethane antiproliferative signaling pathways control cell-cycle gene transcription in human breast cancer cells by regulating promoter-Sp1 transcription factor interactions. J Nutr 2003;133**:**2448S-55S.
15. Nachshon-Kedmi M, Yannai S, Haj A, Fares FA. Indole-3-carbinol and 3,3'-diindolylmethane induce apoptosis in human prostate cancer cells. Food Chem Toxicol 2003;41**:**745-52.
16. Hong C, Kim HA, Firestone GL, Bjeldanes LF. 3,3'-Diindolylmethane (DIM) induces a G(1) cell cycle arrest in human breast cancer cells that is accompanied by Sp1-mediated activation of p21(WAF1/CIP1) expression. Carcinogenesis 2002;23**:**1297-305.
17. Rahman KM, Li Y, Sarkar FH. Inactivation of akt and NF-kappaB play important roles during indole-3-carbinol-induced apoptosis in breast cancer cells. Nutr Cancer 2004;48**:**84-94.
18. Chinni SR, Li Y, Upadhyay S, Koppolu PK, Sarkar FH. Indole-3-carbinol (I3C) induced cell growth inhibition, G1 cell cycle arrest and apoptosis in prostate cancer cells. Oncogene 2001;20**:**2927-36.
19. Sarkar FH, Li Y. Indole-3-carbinol and prostate cancer. J Nutr 2004;134**:**3493S-8S.
20. Le HT, Schaldach CM, Firestone GL, Bjeldanes LF. Plant-derived 3,3'-Diindolylmethane is a strong androgen antagonist in human prostate cancer cells. J Biol Chem 2003;278**:**21136-45.
21. Anderton MJ, Manson MM, Verschoyle R et al. Physiological modeling of formulated and crystalline 3,3'-diindolylmethane pharmacokinetics following oral administration in mice. Drug Metab Dispos 2004;32**:**632-8.
22. Chaturvedi MM, Mukhopadhyay A, Aggarwal BB. Assay for redox-sensitive transcription factor. Methods Enzymol 2000;319**:**585-602.
23. Koivisto P, Kononen J, Palmberg C et al. Androgen receptor gene amplification: a possible molecular mechanism for androgen deprivation therapy failure in prostate cancer. Cancer Res 1997;57**:**314-9.
24. Visakorpi T, Hyytinen E, Koivisto P et al. In vivo amplification of the androgen receptor gene and progression of human prostate cancer. Nat Genet 1995;9**:**401-6.
25. Chen CD, Welsbie DS, Tran C et al. Molecular determinants of resistance to antiandrogen therapy. Nat Med 2004;10**:**33-9.
26. Wang L, Ossowski L, Ferrari AC. Overexpressed Androgen Receptor Linked to p21WAF1 Silencing May Be Responsible for Androgen Independence and Resistance to Apoptosis of a Prostate Cancer Cell Line. Cancer Res 2001;61**:**7544-51.
27. Chatterjee B. The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer. Mol Cell Biochem 2003;253**:**89-101.

28. Sharma M, Chuang WW, Sun Z. Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3beta inhibition and nuclear beta-catenin accumulation. J Biol Chem 2002;277**:**30935-41.
29. Lee SO, Lou W, Nadiminty N, Lin X, Gao AC. Requirement for NF-(kappa)B in interleukin-4-induced androgen receptor activation in prostate cancer cells. Prostate 2005;64**:**160-7.
30. Majumder PK, Sellers WR. Akt-regulated pathways in prostate cancer. 0 AD;24**:**7465-74.
31. Wang L, Lin HK, Hu YC, Xie S, Yang L, Chang C. Suppression of androgen receptor-mediated transactivation and cell growth by the glycogen synthase kinase 3 beta in prostate cells. J Biol Chem 2004;279**:**32444-52.
32. Yang L, Xie S, Jamaluddin M et al. Induction of Androgen Receptor Expression by Phosphatidylinositol 3-Kinase/Akt Downstream Substrate, FOXO3a, and Their Roles in Apoptosis of LNCaP Prostate Cancer Cells. J Biol Chem 2005;280**:**33558-65.
33. Sweeney C, Li L, Shanmugam R et al. Nuclear Factor-{kappa}B Is Constitutively Activated in Prostate Cancer In vitro and Is Overexpressed in Prostatic Intraepithelial Neoplasia and Adenocarcinoma of the Prostate. Clin Cancer Res 2004;10**:**5501-7.
34. Zerbini LF, Wang Y, Cho JY, Libermann TA. Constitutive activation of nuclear factor kappaB p50/p65 and Fra-1 and JunD is essential for deregulated interleukin 6 expression in prostate cancer. Cancer Res 2003;63**:**2206-15.
35. Suh J, Payvandi F, Edelstein LC et al. Mechanisms of constitutive NF-kappaB activation in human prostate cancer cells. Prostate 2002;52**:**183-200.
36. Palvimo JJ, Reinikainen P, Ikonen T, Kallio PJ, Moilanen A, Janne OA. Mutual transcriptional interference between RelA and androgen receptor. J Biol Chem 1996;271**:**24151-6.
37. Chen CD, Sawyers CL. NF-kappa B activates prostate-specific antigen expression and is upregulated in androgen-independent prostate cancer. Mol Cell Biol 2002;22**:**2862-70.
38. Fenton MA, Shuster TD, Fertig AM et al. Functional characterization of mutant androgen receptors from androgen-independent prostate cancer. Clin Cancer Res 1997;3**:**1383-8.
39. Taplin ME, Bubley GJ, Shuster TD et al. Mutation of the androgen-receptor gene in metastatic androgen-independent prostate cancer. N Engl J Med 1995;332**:**1393-8.
40. Ozes ON, Mayo LD, Gustin JA, Pfeffer SR, Pfeffer LM, Donner DB. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature 1999;401**:**82-5.

FOOTNOTES

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The abbreviations used are: DIM, 3,3'-diindolylmethane; B-DIM, formulated 3,3'-diindolylmethane from BioResponse; AR, androgen receptor; PI3K, phosphatidylinositol 3(OH)-kinase; FOXO, Forkhead transcription factors; NF-κB, nuclear factor-kappa B; PSA, prostate-specific antigen; ARE, androgen-responsive element; FBS, fetal bovine serum; DCC-FBS, dextran-coated charcoal-stripped fetal bovine serum; DHT, dihydrotestosterone; EMSA, electrophoretic mobility shift assay; HRPC, hormone-refractory prostate cancer.

FIGURE LEGENDS

Fig. 1. **B-DIM significantly inhibited cell growth and induced apoptosis in LNCaP and C4-2B cells.** For cell growth inhibition assay, LNCaP (A) and C4-2B (B) cells were treated with 0-50 μM B-DIM for 48 and 72 hours. Cell viability was tested by MTT assay. For apoptosis assay, LNCaP (C) and C4-2B (D) cells were treated with 10 or 25 μM B-DIM for 24, 48, and 72 hours. Apoptotic cell death was tested by Histone/DNA ELISA. (*: $p<0.05$ compared to control, n=3)

Fig. 2. **B-DIM significantly inhibited expression of AR and PSA at mRNA and protein levels in LNCaP cells.** A. Western Blot analysis showed that B-DIM inhibited expression of AR and PSA protein in time- (upper panel) and dose- (lower panel) dependent manners. B. LNCaP cells were treated with 10 and 25 μM B-DIM for 24 hours followed by 0.1 and 1 nM DHT treatment for 2 hours. Western Blot analysis showed that both 10 μM (upper panel) and 25 μM (lower panel) B-DIM abrogated the expression of AR and PSA protein stimulated by DHT. C. Real-time RT-PCR showed that 10 and 25 μM B-DIM inhibited AR mRNA expression stimulated by 0.1 and 1 nM DHT. D. Real-time RT-PCR showed that 10 and 25 μM B-DIM inhibited PSA mRNA expression stimulated by 0.1 and 1 nM DHT.

Fig. 3. **B-DIM significantly inhibited expression of AR and PSA at mRNA and protein levels in C4-2B cells.** A. Western Blot analysis showed that B-DIM inhibited expression of AR and PSA protein in time- (upper panel) and dose- (lower panel) dependent manners. B. C4-2B cells were treated with 10 and 25 μM B-DIM for 24 hours followed by 0.1 and 1 nM DHT treatment for 2 hours. Western Blot analysis showed that both 10 μM (upper panel) and 25 μM (lower panel) B-DIM abrogated the expression of AR and PSA protein stimulated by DHT. C. Real-time RT-PCR showed that 10 and 25 μM B-DIM inhibited AR mRNA expression stimulated by 0.1 and 1 nM DHT. D. Real-time RT-PCR showed that 10 and 25 μM B-DIM inhibited PSA mRNA expression stimulated by 0.1 and 1 nM DHT.

Fig. 4. **B-DIM did not inhibit expression of AR in AR stably transfected PC-3 cells but it significantly inhibited the activity of PSA promoter in LNCaP and C4-2B cells.** A. B-DIM did not inhibit AR protein expression in AR stably transfected PC-3 cells. AR stably transfected PC-3 cells were treated with 10 and 25 μM B-DIM for 24 hours followed by 0.1 and 1 nM DHT treatment for 2 hours. Western Blot analysis showed that both 10 and 25 μM B-DIM did not inhibit the AR expression stimulated by DHT. B and C. LNCaP (B) and C4-2B (C) cells were transiently co-transfected with PSA-Luc and pSV-β-gal by ExGen 500. After 5 hours, the transfected cells were washed and incubated with complete RPMI-1640 medium overnight followed by the treatment with 10 and 25 μM B-DIM for 24 and 48 hours. Subsequently, the luciferase and β-galactosidase activities in the samples were measured. The comparative luciferase activity were calculated and adjusted by β-galactosidase activity. (*: $p<0.05$ compared to PSA-Luc transfection, n=3)

Fig. 5. **B-DIM inhibited the expression of p-Akt, nuclear NF-κB, AR, and PSA in Akt transfected C4-2B and LNCaP cells.** C4-2B and LNCaP cells were transiently co-transfected with NF-κB-Luc and pLNCX-Akt (wild type Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control empty vector). After 5 hours, the transfected cells were washed and incubated with complete RPMI-1640 medium overnight followed by treatment with 50 μM B-DIM for 48 hours. Subsequently, the cells were

lysed and Western Blot analysis was conducted to test the expression of Akt, p-Akt, AR, PSA, and NF-κB. (Wild Akt: wild type Akt; Mut Akt: dominant negative Akt; Myr Akt: constitutively activated Akt; Emp: control empty vector.)

Fig. 6. **B-DIM inhibited NF-κB DNA binding activity in C4-2B and LNCaP cells.** A. B-DIM inhibited NF-κB DNA binding activity in Akt transfected C4-2B and LNCaP cells tested by luciferase assay. C4-2B and LNCaP cells were transiently co-transfected with NF-κB-Luc and pLNCX-Akt (wild type Akt), pLNCX-Myr-Akt (constitutively activated Akt), pLNCX-Akt-K179M (dominant negative), and pLNCX (control empty vector). After 5 hours, the transfected cells were washed and incubated with complete RPMI-1640 medium overnight followed by treatment with 50 μM B-DIM for 48 hours. Subsequently, the luciferase activity in the transfected cells was tested by luciferase assay. (Wild Akt: wild type Akt; Mut Akt: dominant negative Akt; Myr Akt: constitutively activated Akt; Emp: control empty vector.) B. B-DIM inhibited NF-κB DNA binding activity in Akt transfected C4-2B and LNCaP cells tested by EMSA. C. B-DIM significantly inhibited NF-κB DNA binding activity stimulated by DHT in LNCaP and C4-2B cells. LNCaP and C4-2B cells were treated with 10 and 25 μM B-DIM for 24 hours followed by 0.1 and 1 nM DHT treatment for 2 hours. Nuclear protein was extracted. EMSA showed that both 10 and 25 μM B-DIM abrogated the activation of NF-κB DNA binding activity stimulated by DHT in LNCaP and C4-2B cells.

Fig. 7. **B-DIM significantly inhibited AR nuclear translocation in LNCaP and C4-2B cells.** A and B. LNCaP (A) and C4-2B cells (B) were treated with 25 μM B-DIM for 24 hours followed by 1 nM DHT treatment for 2 hours. Cells were then incubated with Anti-AR monoclonal antibody followed by incubation with FITC-conjugated anti-mouse antibody along with 0.1 μg/ml DAPE. Cell images were captured on a confocal microscope system, using 63 X 1.2 objective and 488/364 nm laser wavelengths to detect FITC and DAPI, respectively. (a and b: LNCaP cell control; c and d: LNCaP cells treated with 25 μM B-DIM; e and f: C4-2B cell control; g and f: C4-2B cells treated with 25 μM B-DIM.) C. Crosstalk of Akt, NF-κB, and PSA with AR and the effects of B-DIM on the crosstalk.

Figure 1

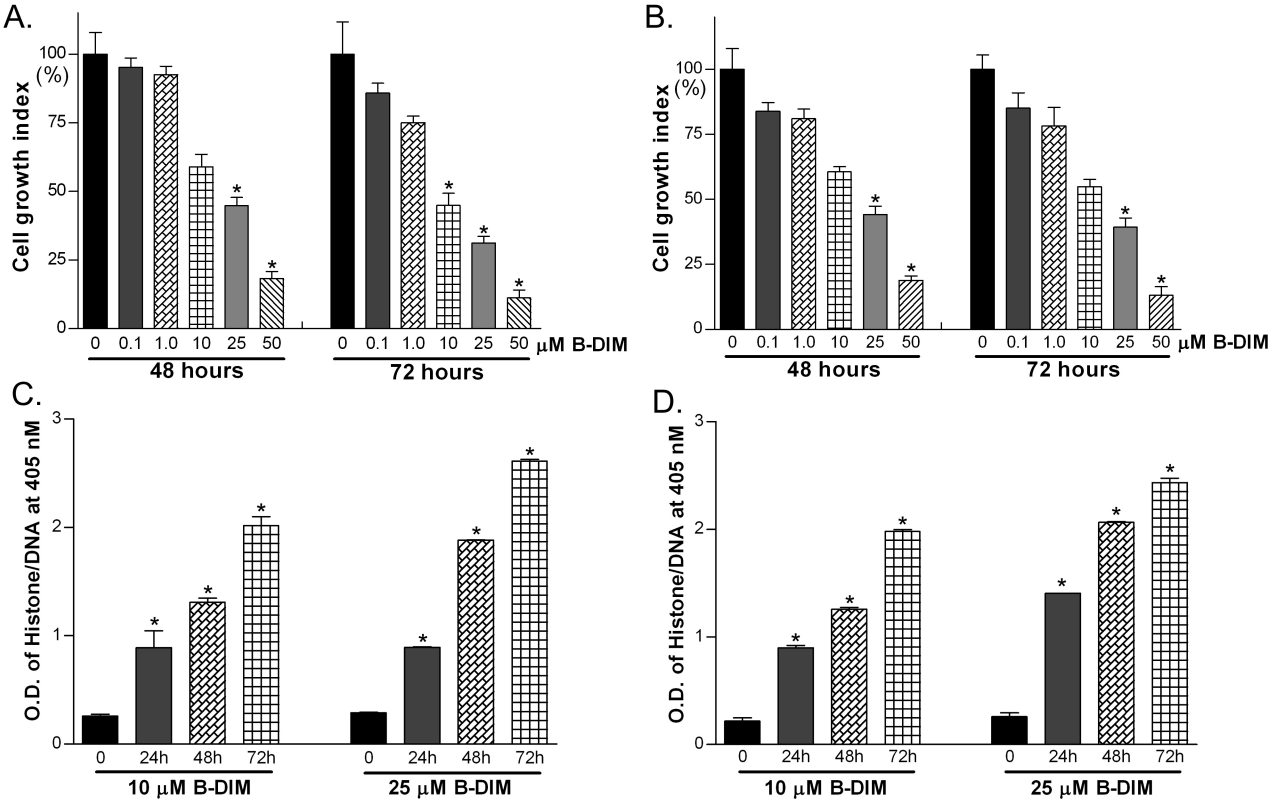

A.
Cell growth index (%)
100
75
50
25
0
0 0.1 1.0 10 25 50 μM B-DIM
48 hours
72 hours
B.
Cell growth index (%)
100
75
50
25
0
0 0.1 1.0 10 25 50 μM B-DIM
48 hours
72 hours
C.
O.D. of Histone/DNA at 405 nM
3
2
1
0
0 24h 48h 72h
10 μM B-DIM
25 μM B-DIM
D.
O.D. of Histone/DNA at 405 nM
3
2
1
0
0 24h 48h 72h
10 μM B-DIM
25 μM B-DIM

Figure 2

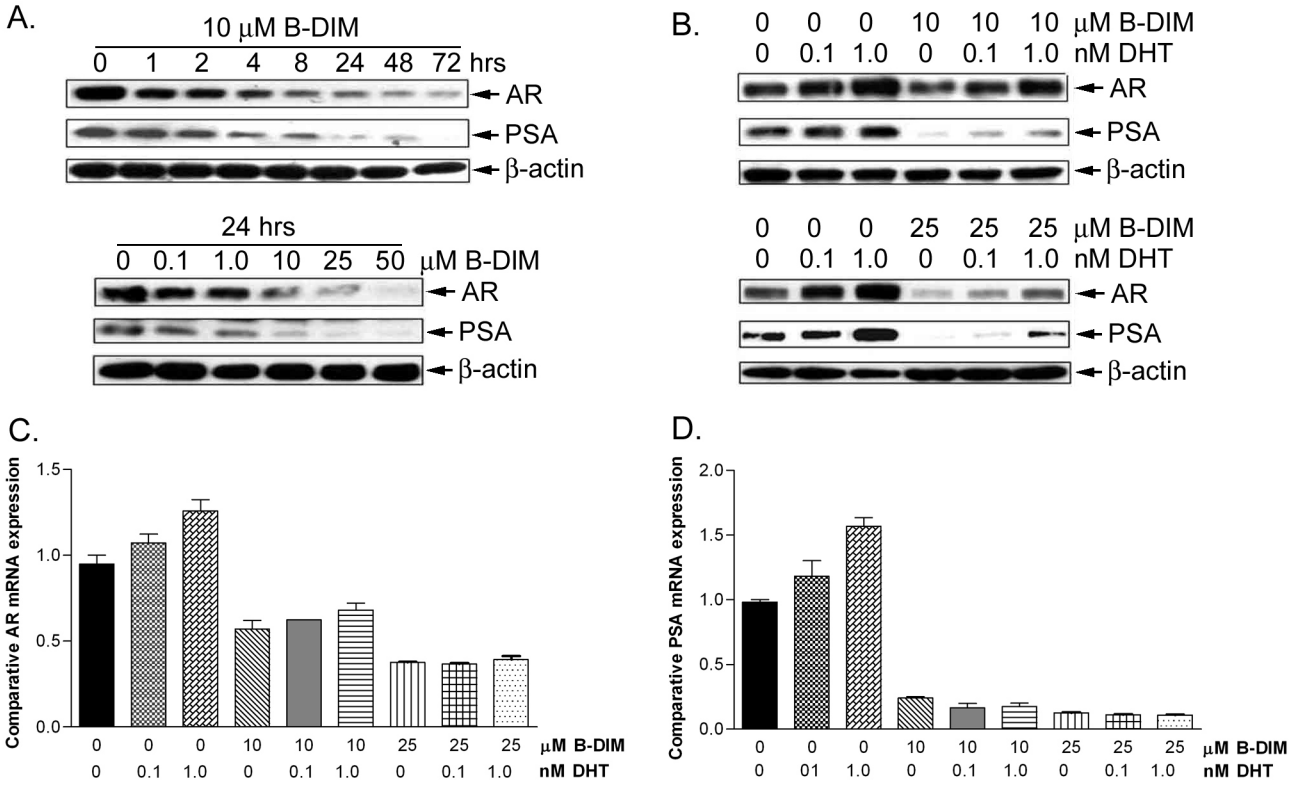
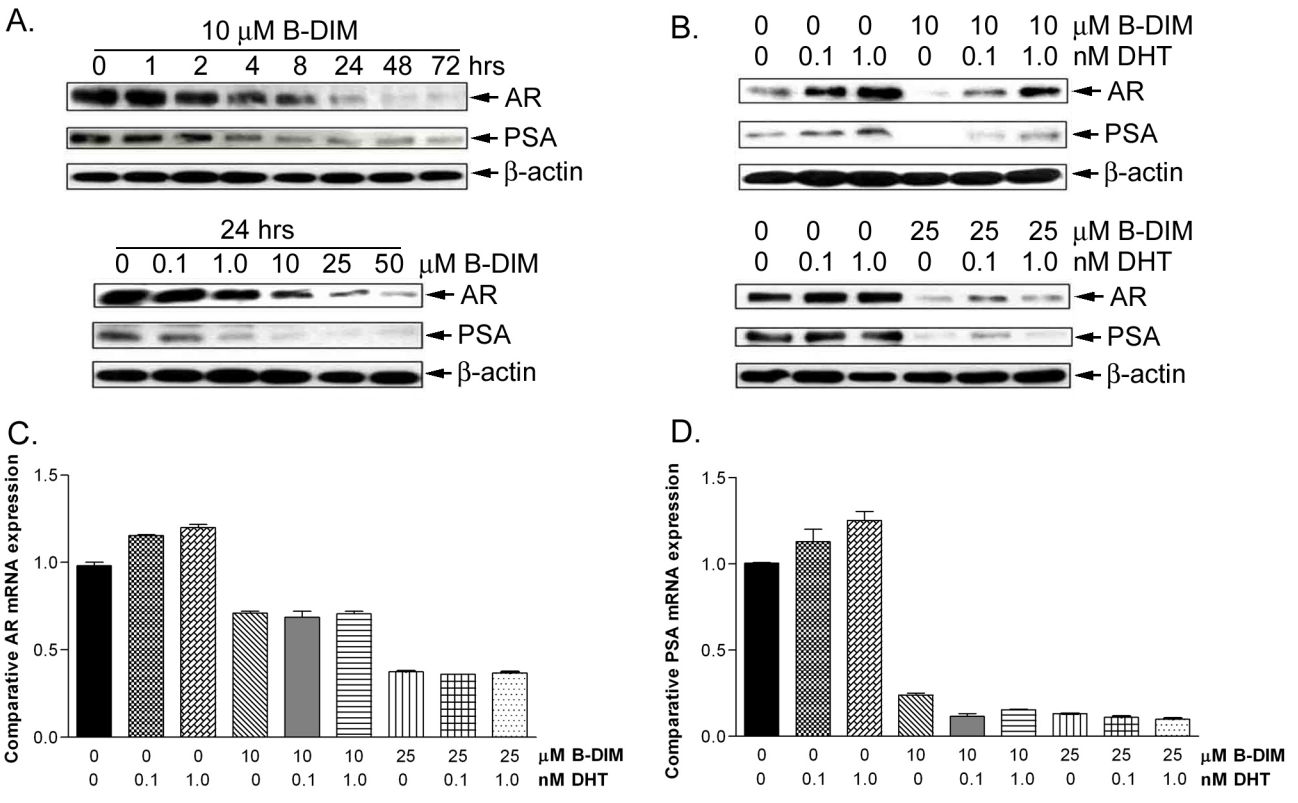

A.
10 μM B-DIM
0 1 2 4 8 24 48 72 hrs
AR
PSA
β-actin
24 hrs
0 0.1 1.0 10 25 50 μM B-DIM
AR
PSA
β-actin
B.
0 0 0 10 10 10 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
AR
PSA
β-actin
0 0 0 25 25 25 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
AR
PSA
β-actin
C.
Comparative AR mRNA expression
1.5
1.0
0.5
0.0
0 0 0 10 10 10 25 25 25 μM B-DIM
0 0.1 1.0 0 0.1 1.0 0 0.1 1.0 nM DHT
D.
Comparative PSA mRNA expression
2.0
1.5
1.0
0.5
0.0
0 0 0 10 10 10 25 25 25 μM B-DIM
0 01 1.0 0 0.1 1.0 0 0.1 1.0 nM DHT

Figure 3


A.
10 μM B-DIM
0 1 2 4 8 24 48 72 hrs
AR
PSA
β-actin
24 hrs
0 0.1 1.0 10 25 50 μM B-DIM
AR
PSA
β-actin
B.
0 0 0 10 10 10 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
AR
PSA
β-actin
0 0 0 25 25 25 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
AR
PSA
β-actin
C.
Comparative AR mRNA expression
1.5
1.0
0.5
0.0
0 0 0 10 10 10 25 25 25 μM B-DIM
0 0.1 1.0 0 0.1 1.0 0 0.1 1.0 nM DHT
D.
Comparative PSA mRNA expression
1.5
1.0
0.5
0.0
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0 0.1 1.0 0 0.1 1.0 0 0.1 1.0 nM DHT

Figure 4

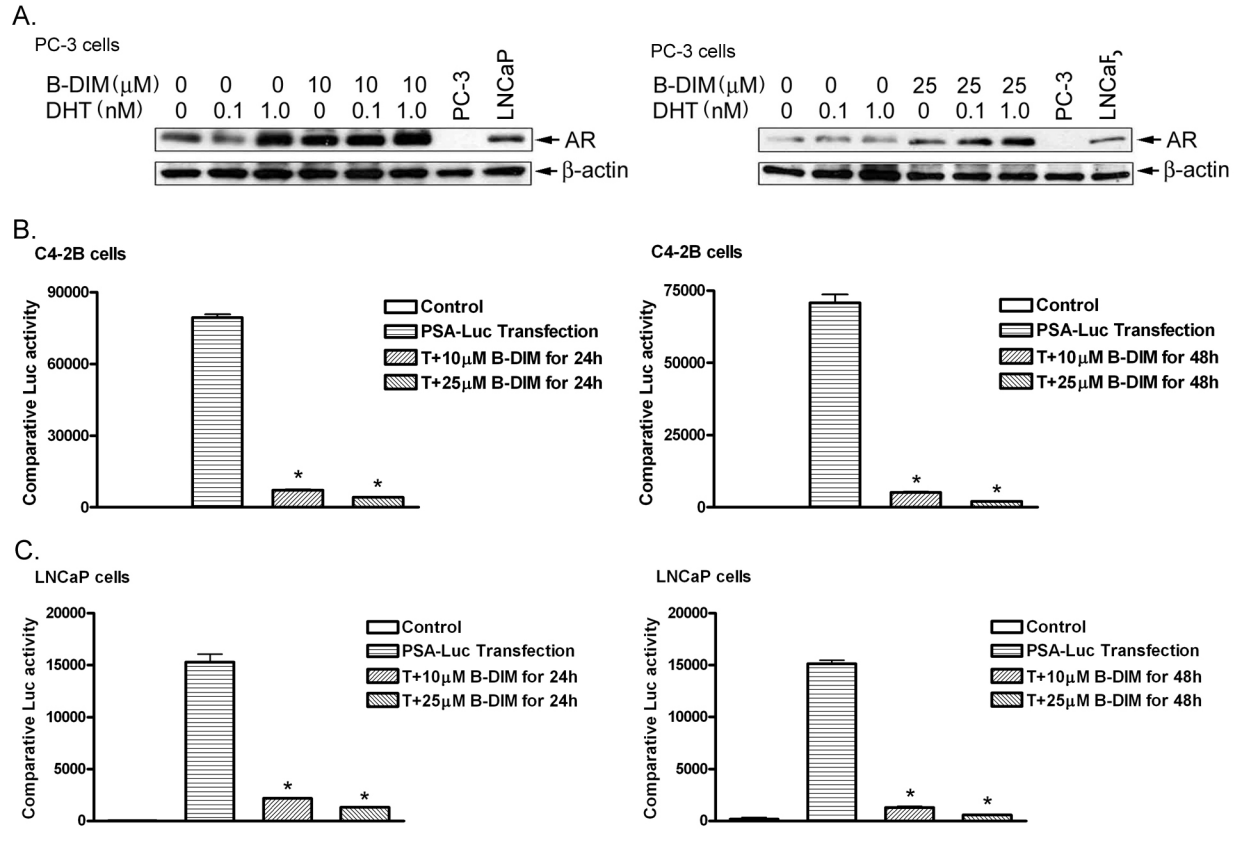
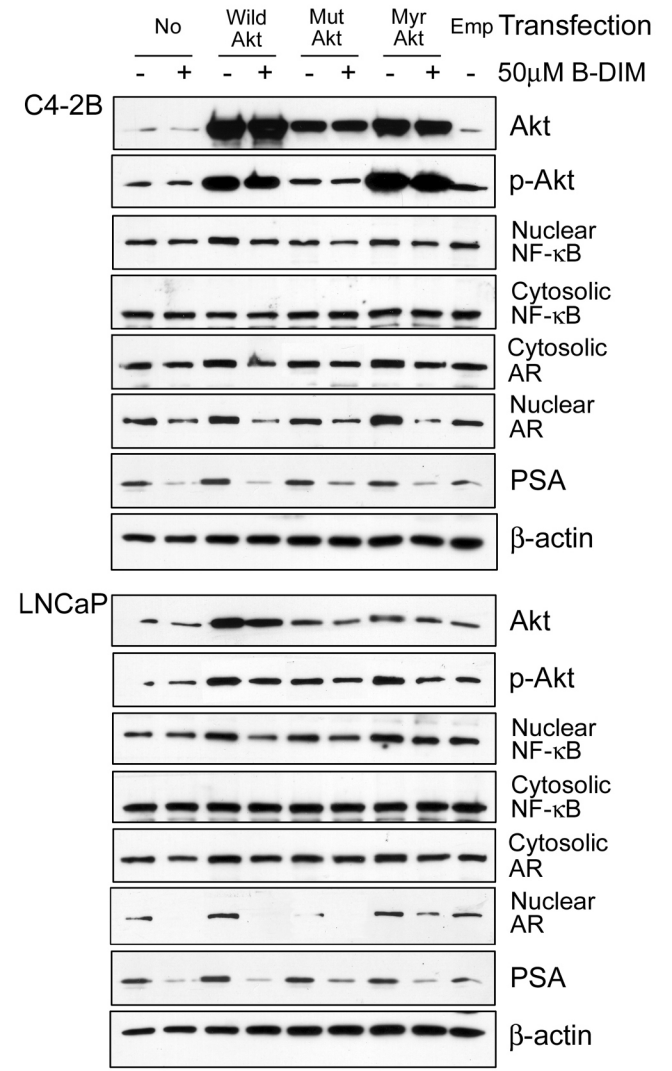

A.
PC-3 cells
B-DIM(μM) 0 0 0 10 10 10
DHT (nM) 0 0.1 1.0 0 0.1 1.0
PC-3
LNCaP
AR
β-actin
PC-3 cells
B-DIM(μM) 0 0 0 25 25 25
DHT (nM) 0 0.1 1.0 0 0.1 1.0
PC-3
LNCaP
AR
β-actin
B.
C4-2B cells
Comparative Luc activity
90000
60000
30000
0
Control
PSA-Luc Transfection
T+10μM B-DIM for 24h
T+25μM B-DIM for 24h
*
*
C4-2B cells
Comparative Luc activity
75000
50000
25000
0
Control
PSA-Luc Transfection
T+10μM B-DIM for 48h
T+25μM B-DIM for 48h
*
*
C.
LNCaP cells
Comparative Luc activity
20000
15000
10000
5000
0
Control
PSA-Luc Transfection
T+10μM B-DIM for 24h
T+25μM B-DIM for 24h
*
*
LNCaP cells
Comparative Luc activity
20000
15000
10000
5000
0
Control
PSA-Luc Transfection
T+10μM B-DIM for 48h
T+25μM B-DIM for 48h
*
*

Figure 5


No
Wild Akt
Mut Akt
Myr Akt
Emp
Transfection
- + - + - + - + -
50μM B-DIM
C4-2B
Akt
p-Akt
Nuclear NF-κB
Cytosolic NF-κB
Cytosolic AR
Nuclear AR
PSA
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Cytosolic AR
Nuclear AR
PSA
β-actin

Figure 6

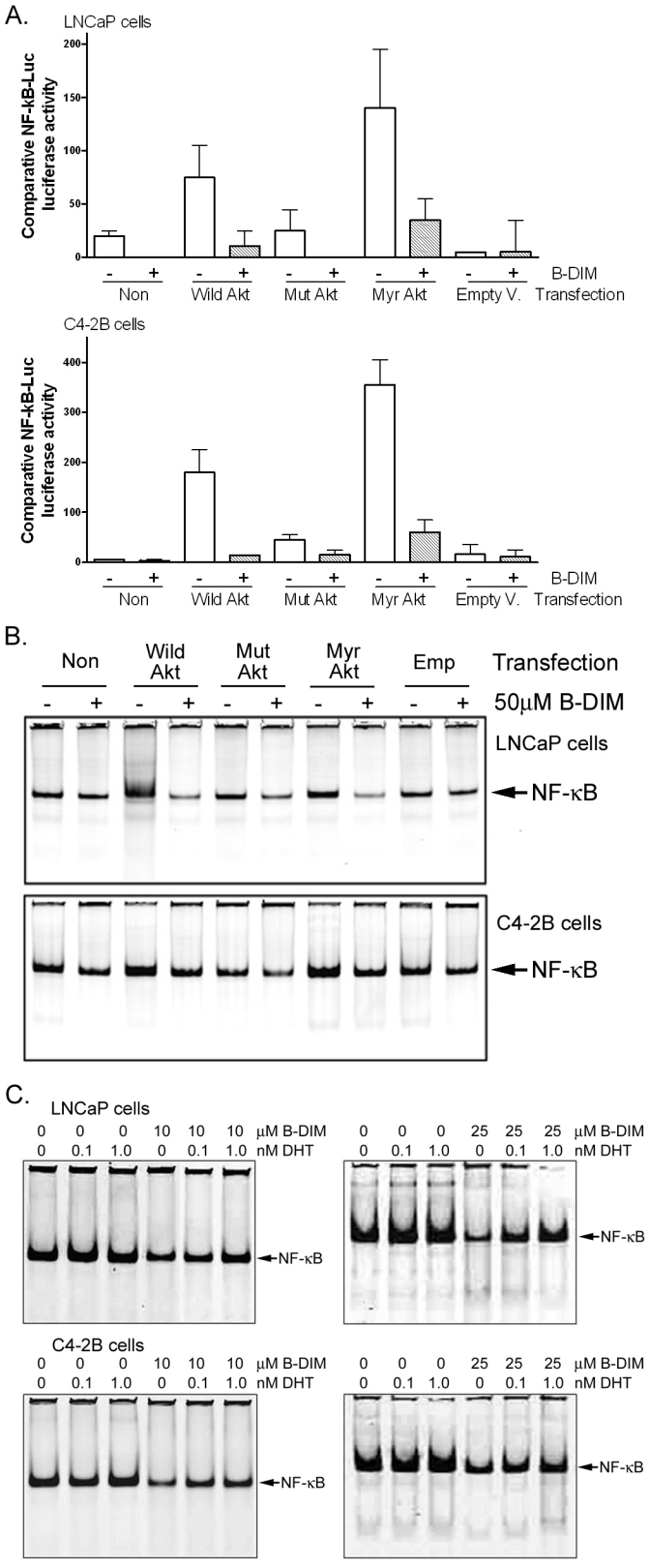
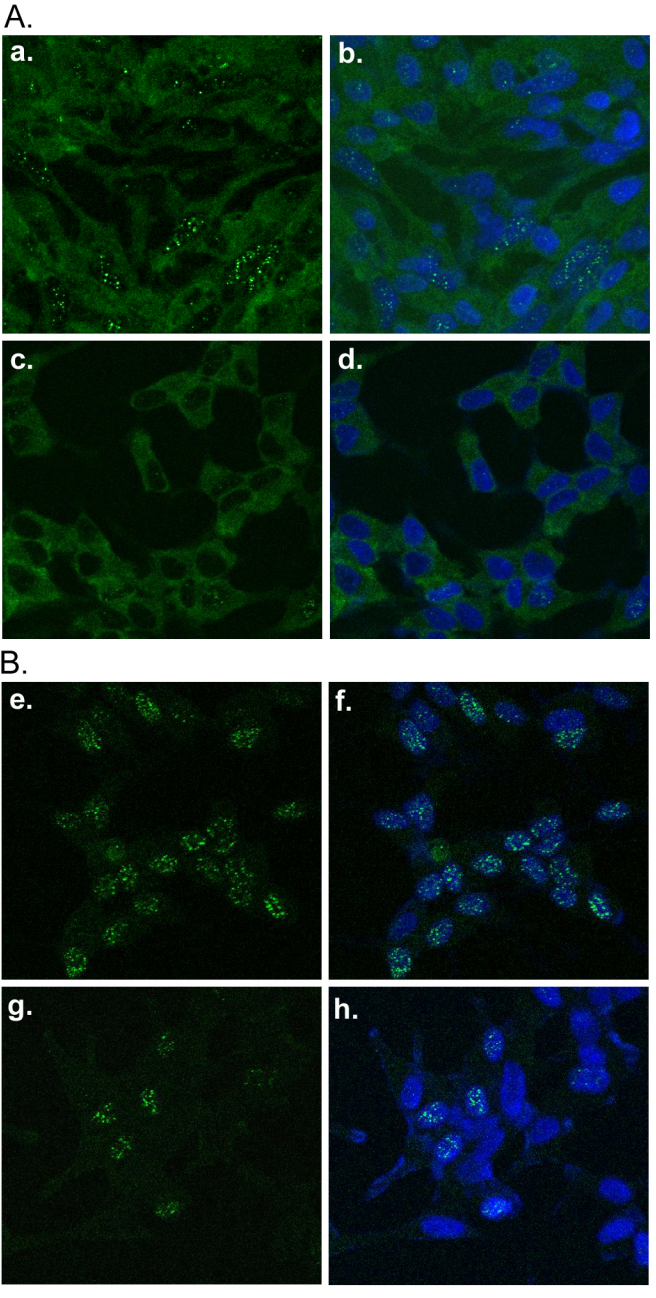
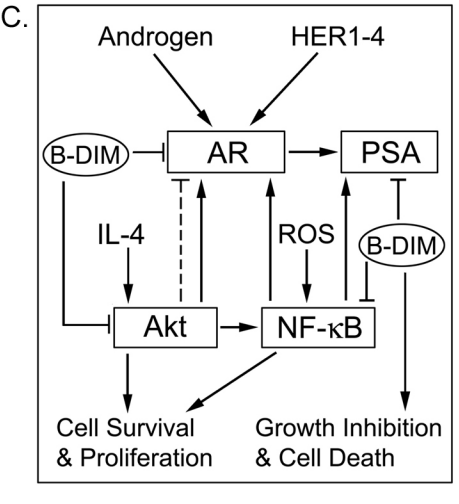

A.
LNCaP cells
Comparative NF-kB-Luc luciferase activity
200
150
100
50
0
- + - + - + - + - +
B-DIM
Non
Wild Akt
Mut Akt
Myr Akt
Empty V.
Transfection
C4-2B cells
Comparative NF-kB-Luc luciferase activity
400
300
200
100
0
- + - + - + - + - +
B-DIM
Non
Wild Akt
Mut Akt
Myr Akt
Empty V.
Transfection
B.
Non
Wild Akt
Mut Akt
Myr Akt
Emp
Transfection
- + - + - + - + - +
50μM B-DIM
LNCaP cells
NF-κB
C4-2B cells
NF-κB
C.
LNCaP cells
0 0 0 10 10 10 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
NF-κB
0 0 0 25 25 25 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
NF-κB
C4-2B cells
0 0 0 10 10 10 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
NF-κB
0 0 0 25 25 25 μM B-DIM
0 0.1 1.0 0 0.1 1.0 nM DHT
NF-κB

Figure 7


A.
a.
b.
c.
d.
B.
e.
f.
g.
h.


C.
Androgen
HER1-4
B-DIM
AR
PSA
IL-4
ROS
B-DIM
Akt
NF-κB
Cell Survival
& Proliferation
Growth Inhibition
& Cell Death